



1-1-2014

Pkc Isozymes in Lung Cancer Development and Therapy Resistance

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Abstract

ABSTRACT

PKC ISOZYMES IN LUNG CANCER DEVELOPMENT AND THERAPY RESISTANCE

Mahlet B. Abera

Marcelo G. Kazanietz, Ph.D.

Non-small cell lung cancer (NSCLC) is one of the major causes of cancer related mortality worldwide and comprises the most frequent type of lung cancers. Oncogenic alterations such as activating mutations in the epidermal growth factor receptor (EGFR) and KRAS have been implicated in the pathogenesis of NSCLC. Protein kinase C (PKC), a family of serine/threonine kinases that are targets of phorbol ester/diacylglycerol, has been shown to be involved in EGFR and KRAS signaling, indicating their importance in lung cancer progression. In this thesis work, we established the requirement of PKC α and PKC ϵ in the malignancy of lung cancer in the context of EGFR and KRAS mutations. Using an isogenic model system, we found NSCLC cells that are resistant to the EGFR-tyrosine kinase inhibitor erlotinib display remarkably high levels of PKC α compared to the parental erlotinib sensitive cells. Inhibition of PKC α resulted in sensitization to erlotinib treatment and loss of gene expression associated with mesenchymal phenotype. Additionally, we showed that PKC α signaling controls activation of a key regulator of lung cancer progression, NF- κ B. PKC α depletion by RNA interference in lung cancer cells indeed attenuates I κ B α phosphorylation, subsequent impairment of NF- κ B translocation into the nucleus, and induction of NF- κ B target genes in response to interleukin 1 beta (IL-1 β). Finally, in the context of KRAS mutation, we presented evidence showing the requirement of PKC ϵ signaling for the formation of KRAS-driven lung tumors in vivo. Tumor formation in the double transgenic KRASG12D;PKC ϵ ^{-/-} mice was remarkably reduced compared to KRASG12D;PKC ϵ ^{+/+} mice. All together, this thesis work highlights the multiple roles that PKC isozymes play in the initiation and progression of lung cancer, and suggests a potential therapeutic role for PKC α and PKC ϵ inhibitors for prevention and treatment of lung tumors.

Degree Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Graduate Group

Pharmacology

First Advisor

Marcelo G. Kazanietz

Keywords

Drug resistance, Inflammation, Lung Cancer, Protein Kinase C, Tumorigenesis

Subject Categories

Oncology | Pharmacology

**PKC ISOZYMES IN LUNG CANCER DEVELOPMENT AND THERAPY
RESISTANCE**

Mahlet B. Abera

A DISSERTATION

in

Pharmacology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2014

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DEDICATION

To my mother Bizunesh Worku and my father Berhanu Abera who always believed in me and did everything they could so that I have a brighter future.

To my sisters Kalkidan and Maedot, and my brother Bisrat for teaching me how to love.

I love you all.

ACKNOWLEDGMENT

The completion of my Ph.D. thesis would not have been possible without the support of many people. I would like to express my gratitude to my thesis advisor Dr. Marcelo Kazanietz whose support and invaluable suggestions made this work successful. Marcelo has given me ample opportunities to get experience in different areas of research and I am very grateful.

I would like to express my deepest appreciation to my thesis committee chair Dr. Judy Meinkoth whose guidance has been essential for my success as a graduate student. I would also like to thank my past and present committee members Dr. Xiaolu Yang, Dr. Jeff Field, Dr. Costas Koumenis, Dr. Emer Smyth and Dr. Joe Kissil whose suggestions and critical evaluation of my work has helped me grow as a scientist.

I have had the pleasure of working with great minds in the Kazanietz lab. Thank you for guiding me through the ups and downs and made lab work more enjoyable. Special thanks to my friends at Penn, across the country and all over the world for the encouragement, fun and priceless moments during graduate school.

Finally, I would like to express my love and gratitude to my beloved families for their support, understanding and endless love through the duration of my studies.

ABSTRACT

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Non-small cell lung cancer (NSCLC) is one of the major causes of cancer related mortality worldwide and comprises the most frequent type of lung cancers. Oncogenic alterations such as activating mutations in the epidermal growth factor receptor (EGFR) and KRAS have been implicated in the pathogenesis of NSCLC. Protein kinase C (PKC), a family of serine/threonine kinases that are targets of phorbol ester/diacylglycerol, has been shown to be involved in EGFR and KRAS signaling, indicating their importance in lung cancer progression. In this thesis work, we established the requirement of PKC α and PKC ϵ in the malignancy of lung cancer in the context of EGFR and KRAS mutations. Using an isogenic model system, we found NSCLC cells that are resistant to the EGFR-tyrosine kinase inhibitor erlotinib display remarkably high levels of PKC α compared to the parental erlotinib sensitive cells. Inhibition of PKC α resulted in sensitization to erlotinib treatment and loss of gene expression associated with mesenchymal phenotype. Additionally, we showed that PKC α signaling controls activation of a key regulator of lung cancer progression, NF- κ B. PKC α depletion by RNA interference in lung cancer

cells indeed attenuates I κ B α phosphorylation, subsequent impairment of NF- κ B translocation into the nucleus, and induction of NF- κ B target genes in response to interleukin 1 beta (IL-1 β). Finally, in the context of KRAS mutation, we presented evidence showing the requirement of PKC ϵ signaling for the formation of KRAS-driven lung tumors *in vivo*. Tumor formation in the double transgenic $KRAS^{G12D};PKC\epsilon^{-/-}$ mice was remarkably reduced compared to $KRAS^{G12D};PKC\epsilon^{+/+}$ mice. All together, this thesis work highlights the multiple roles that PKC isozymes play in the initiation and progression of lung cancer, and suggests a potential therapeutic role for PKC α and PKC ϵ inhibitors for prevention and treatment of lung tumors.

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CHAPTER 1: Introduction

1.1 Lung cancer

Lung cancer is the largest contributor to new cancer diagnosis and cancer-related death each year [57]. Most lung cancer patients have advanced disease at the time of diagnosis, shortening the 5-year survival rate to only 10-15 %. On the basis of histology, lung cancers are classified as small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC comprises 80 % of lung cancer cases and is further subdivided into adenocarcinomas (50 %), squamous cell carcinoma (35 %) and large cell carcinoma (15 %). Small cell lung carcinomas and squamous cell carcinomas arise from the central compartments of the lung, while adenocarcinomas arise from the progenitor cells of the bronchioles (Clara cells), alveolar type II cells, or from mucin producing cells in the peripheral compartment of the lungs (Table 1.1) [24, 162].

Lung tumorigenesis involves a series of progressive pathological changes in the mucosa membrane lining the respiratory tract. In squamous cell carcinoma development, the bronchial epithelium undergoes a series of morphological changes that represent cell transformation from normal into precursor lesions, including hyperplastic, metaplastic, and dysplastic lesions. While hyperplasia and squamous metaplasia are considered reversible changes, dysplastic lesions are most frequently associated with the development of squamous cell lung carcinomas. Similarly, adenocarcinomas develop as a result of transformation from premalignant precursor lesions to carcinoma *in situ*. However, in the case of adenocarcinomas, the only known precursor lesion is atypical adenomatous hyperplasia [62, 65, 205].

Tumor type	Tumor subtype	Incidence	Location
NSCLC	Adenocarcinoma	40 %	Outer edge of lungs, from mucus producing cells
	Squamous cell carcinoma	25 %	Central compartment, from cells that line the airways
	Large cell carcinoma	15 %	Any area of the lung
SCLC		15 %	In the bronchi near the center of the chest
	Others/mixed	5 %	Mixed

Table 1.1 Histological classification of lung cancer

Lung cancer is commonly classified as small cell carcinoma (SCLC) and a heterogeneous group of non-small cell carcinomas (NSCLC), which includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma. SCLCs arise in the central compartment of the lungs, while NSCLCs commonly arise in the peripheral regions of the lungs.

Tobacco smoke is the main cause of lung cancer with 80-90 % arising in cigarette smokers. However, in spite of the exposure to carcinogens, only 10-15 % of heavy smokers ultimately develop the disease, which reflects the role of genetic factors in predisposing to lung cancer [20]. The concept of field of cancerization has been described to elucidate the implication of tobacco smoke in cancer initiation. Exposure to tobacco smoke creates injury in the airway epithelial cells leading to development of field of cancerization with genetically and epigenetically altered cells. Field of cancerization consists of tissue adjacent to neoplastic lesions that display normal histology, but harbors molecular abnormalities similar to tumors [37]. There are three major theories describing the origin of smoking-related field of cancerization in lung tumorigenesis. One, injury due to cigarette smoke causes a single mutant epithelial cell clone that expands and extends into the respiratory lining. Second, exposure to carcinogens in cigarette smoke damages the entire airway tract and results in random genetic mutations in multiple areas

that arise from polyclonal tumor stem cells. The third theory is that once the tumor is initiated, the cancer itself affects the surrounding tissue, contributing to alterations including oncogenic activations, tumor suppressor gene (TSG) silencing, and widespread loss of heterozygosity leading to malignancy [62, 113, 170].

Key genetic and epigenetic changes in NSCLC

1. Genetic susceptibility

A number of genetic polymorphisms that make smokers susceptible to lung cancer have been identified. For the most part, polymorphisms have been associated with genes encoding proteins involved in the metabolism, detoxification and gene repair due to tobacco smoke carcinogens. Polymorphism in CYP1A1, an enzyme that catalyzes the bioactivation of polycyclic aromatic hydrocarbons (PAH), contributes to tobacco-induced cancers through their enhanced carcinogen-activating capacity [78]. Polymorphism in CYP2E1, an enzyme involved in the activation and metabolism of alcohols and nitrosamines, is also implicated in tobacco-induced carcinogenesis. Using human lung autopsies, studies correlated higher 7-methyl-dGMP adducts caused by tobacco smoke with the presence of CYP2E1 minor alleles [72]. CYP2A6 is another enzyme thought to be responsible for nitrosamine and nicotine metabolism and has been an important target of research on CYP family gene polymorphisms in relation to lung carcinogenesis. Large size clinical studies also indicate that deletion-type polymorphism at the CYP2A6 locus reduces the risk of lung cancer [78].

Similarly polymorphism in genes associated with detoxification of carcinogens are linked to lung cancer development. Incidence of polymorphisms in glutathione S-

transferase genes (GSTM1, GSTT1, GSTP1 or GSTM3) results in defective enzymatic activity that lead to increased accumulation of carcinogens. Such accumulation entails increased rate of PAH DNA adducts as well as higher rate of mutations within specific genes implicated in the cell cycle and differentiation [79]. Moreover, the risk of lung cancer increases dramatically in tumor samples that exhibit combinational polymorphisms [199].

Genetic defect in DNA repair genes is another factor that makes smokers susceptible to lung cancer. Under normal circumstances, DNA repair mechanisms prevent the accumulation of multiple genetic alterations that could potentially lead to cancer development. Specifically, studies linked *hOGG1* polymorphisms to lung cancer incidence both in squamous cell lung carcinoma and adenocarcinomas of the lung [211, 218]. *hOGG1* is a base excision repair protein that removes a mutagenic base product (8-Oxo-2'-deoxyguanosine) that develops from exposure to tobacco smoke, which produces free radicals in the respiratory tract. If repair is inadequate, DNA damage from these free radicals results in mutations predisposing smokers to lung cancer [173].

2. Loss of tumor suppressor genes

Silencing of TSGs is a common event in cancer. TSGs are inactivated in a two-step process that results in the loss of both gene alleles. Initially, mutations or epigenetic silencing inactivates one allele followed by loss of a region of the chromosome by deletion or mitotic recombination through a process called loss of heterozygosity [93]. Common TSGs inactivated in lung cancer include *TP53*, *Rb1*, *P16* and *PTEN*.

The p53 pathway: There is a positive correlation between tobacco use and inactivating mutations in the *p53* TSG [77]. The *p53* gene is located on chromosome 17p13 and

encodes for a 53-kDa phosphoprotein [20]. p53 acts as a transcriptional regulator implicated in the cell cycle, DNA repair, and apoptosis. During cellular stress, it induces the expression of the cyclin-dependent kinase (CDK) inhibitor p21^{cip1}, which results in the G1 cell cycle arrest associated with either DNA repair or apoptosis [36]. p53 was shown to prevent the accumulation of DNA damage by activating DNA repair genes such as *GADD45*. In situations where the damage could not be repaired, p53 promotes the expression of pro-apoptotic genes such as *BAX* and *Fas* [139, 219]. Hemizygous deletion of the *TP53* gene occurs in 65 % of NSCLC, more commonly in squamous cell carcinoma than adenocarcinomas, and it is associated with male gender, higher tumor grade and overall unfavorable prognosis in lung cancer [77, 189].

The p16/CDK-cyclin D/Rb pathway: The retinoblastoma (*Rb*) TSG is located on chromosome 13q14 and encodes a 110-kDa phosphoprotein. pRb prevents the transition from G1 to S phase of the cell cycle by sequestering the transcription factor E2F. This inhibition is however released upon phosphorylation of Rb mediated by complexes of cyclin D and CDK4/CDK6, which enables cells to enter the S phase in the cell cycle [69, 161]. Although alterations in Rb expression are observed in subsets of NSCLC, the most striking is the loss of p16 protein upstream of Rb. p16 inhibits the formation of CDK-cyclin D complexes, thus leading to decreased phosphorylation of Rb. Hence, loss of p16 results in release of E2F upon Rb phosphorylation and progression into S phase of the cell cycle [148]. Loss of p16 function in NSCLC cells is attributed to several mechanisms. NSCLC cells display homozygous or heterozygous deletion within the coding region of p16. Additionally, hypermethylation of the 5'-CpG island in the

promoter region leads to gene silencing [46, 58]. In addition to the loss of Rb and p16 functions, overexpression of cyclin D1 has also been observed in NSCLC [143].

PTEN: Phosphate and tensin homolog (PTEN) is a tumor suppressor gene located on chromosome 10. PTEN removes the phosphate from the PI3K product phosphatidylinositol (3,4,5)-triphosphate (PIP3) to generate phosphoinositide 4,5-biphosphate (PIP2). The decrease in PIP3 attenuates the recruitment of kinases such as phosphoinositide-dependent kinase-1 (PDK-1), which in turn phosphorylates Akt. Loss of PTEN expression leads to continuous Akt activity resulting in cell proliferation and multitude of anti-apoptotic signals [39, 169]. In contrast to other cancers, mutations or homozygous deletions in the *PTEN* gene are not common in NSCLC. However, approximately 70 % of NSCLC have reduced or loss of PTEN expression. These results suggest that the loss of PTEN expression in NSCLC is regulated at the transcriptional or translational level [166, 188].

3. Activation of oncogenes

Unlike inactivation of TSG that involves deletion of both alleles, oncogenes are activated by mechanisms that target one allele and entail gene amplifications or point mutations. Several oncogenic dysregulations have been documented in lung cancer involving KRAS, EGFR, ALK, HER2, MET, PIK3CA, BRAF and ERK (Fig. 1.1). The following introduction only covers the two most commonly mutated oncogenes in NSCLC: KRAS and EGFR.

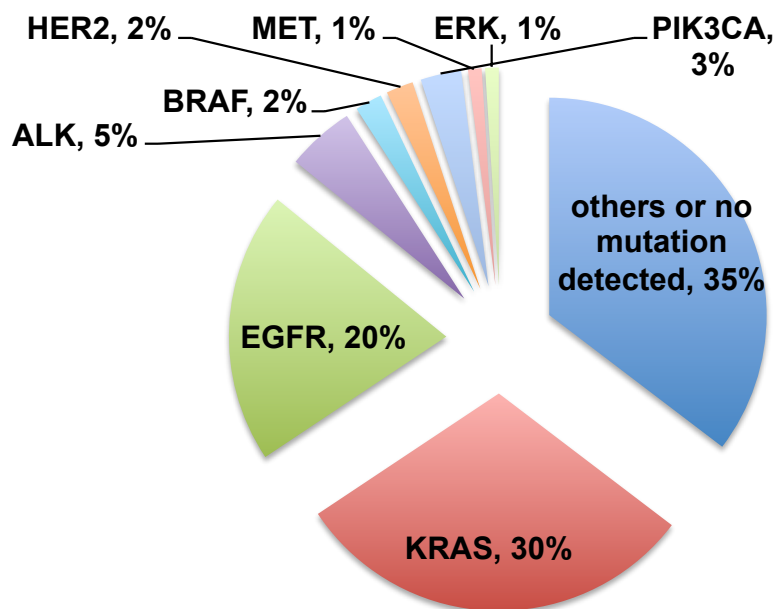


Figure 1.1 Frequency of mutations and amplifications of major oncogenes in NSCLC

Approximately 20 % of lung adenocarcinomas harbor *EGFR* mutations. *KRAS* mutations are more frequently found in adenocarcinomas (30 %), which are mutually exclusive with *EGFR* mutations. A subset of adenocarcinoma harbors a transforming fusion gene, *EML4-ALK* (5 %). The mutation frequency of *BRAF* is 2 %, *PIK3CA* 3 %, and amplifications of *MET* and *HER2* 1 % and 2 %, respectively. Nearly 35 % of lung adenocarcinomas do not harbor currently detectable mutations.

KRAS: Mutations in the *KRAS* gene have been found in approximately one-third of lung adenocarcinoma patients. *KRAS* is a member of the RAS family of small GTPases, which also includes *NRAS* and *HRAS*. The RAS proteins contain a highly conserved N-terminal domain implicated in GTP binding, hydrolysis, and interaction with downstream effectors. On the other hand, the C-terminus contains domains that are highly variable and important for post-translational modifications [202]. Activation of RAS begins upon stimulation of upstream receptors such as receptor tyrosine-kinases (RTKs), which leads

to interaction of adapter proteins such as Grb2 with the intracellular domain of RTKs, and in turn recruits guanine nucleotide exchange factors (GEFs) such as SOS. GEFs interact with RAS to promote the exchange of GDP to GTP. Once bound to GTP, RAS proteins activate a large number of downstream effectors implicated in proliferation (*e.g.* RAF) and survival (*e.g.* PI3K) [41, 87].

KRAS is the most commonly mutated RAS protein in lung cancer. KRAS mutations impair the intrinsic GTPase activity resulting in continuously activated GTP bound KRAS. Most mutations occur in exon 2 and 3, mainly at codon 12, sometimes at codon 13, and more rarely at codon 61 [41, 153]. KRAS mutations are mainly seen in adenocarcinomas of the lung with a history of smoking. The common nucleotide transversions in lung adenocarcinoma (G to T) are frequent in KRAS mutations and are associated with smoking [150].

Meta-analysis of various studies indicates that KRAS mutations are negative prognostic factors for survival of lung cancer patients [132]. It has been reported that NSCLC patients with wild-type KRAS respond better to chemotherapeutic agents such as cisplatin and vinorelbine compared to patients with mutant KRAS [52, 174]. Additionally, KRAS mutations, which are mutually exclusive with EGFR mutations, are associated with lack of response to EGFR tyrosine-kinase inhibitors [180, 213]. Some studies also reported a lack of significant correlation between KRAS status and disease-free progression of NSCLC patients. It has been suggested that rather than having a wild-type KRAS or mutant KRAS, specific types of mutations are better predictors in determining response to therapy and prognosis [174].

The Epidermal Growth Factor Receptor (EGFR): Alterations of EGFR are implicated in the pathogenesis of many tumors, including NSCLC. EGFR encodes an extracellular ligand-binding domain, a transmembrane domain, and an intracellular region consisting of a highly conserved tyrosine-kinase domain. Ligand binding induces homodimerization or heterodimerization of the receptor with other members of the EGFR family, resulting in autophosphorylation of the tyrosine residues. These phosphorylated tyrosine residues provide a docking site for SH2 (Src homology) or PTB (phosphotyrosine binding) domain containing adaptor proteins. EGFR is involved in the regulation of numerous cellular processes such as cell proliferation, survival, differentiation, invasion and metastasis. Overexpression of EGFR and its ligand, the transforming growth factor alpha (TGF α), or activating mutations in EGFR, lead to activation of signaling pathways including the RAS/RAF/MEK/ERK pathway, the PI3K/Akt/mTOR pathway, and the stress activated pathway involving JAK and STAT [140].

Overexpression of EGFR or its ligand TGF α occurs in 50-80 % of NSCLC, specifically in squamous cell carcinomas and its precursor lesions whereas EGFR activating mutation are more frequent in adenocarcinomas, women, East Asians background, and people who never smoked. Activating mutations of the EGFR have been reported in 15-50 % of NSCLC patients (15 % in the case of Caucasian patients and 50 % of East Asian patients). These mutations are often oncogenic and force the mutated cells to become addicted on EGFR for survival [95, 187, 213, 215]. Several activating mutations in the *EGFR* gene have been described. The two most common are a short-

frame deletion of exon 19 (delE746-A750) and a point mutation (CTG to CGG) in exon 21 that results in substitution of leucine by arginine at codon 858 (L858R) [94].

EGFR targeted therapies for NSCLC

Monoclonal antibodies to the EGFR and small molecule tyrosine-kinase inhibitors are in clinical use for lung cancer patients. Monoclonal antibodies including cetuximab, matuzumab, panitumumab, and necitumumab bind to the extracellular domain of EGFR and competitively block ligand binding. The antibody-receptor complex formation is followed by internalization and degradation of the complex, leading to downregulation of EGFR signaling [57, 85]. The small molecule EGFR TKIs such as gefitinib and erlotinib compete reversibly with ATP for binding to the intracellular catalytic kinase domain. The efficiency of these drugs for blocking receptor activity and decreasing cell viability is significantly higher in cells harboring mutant EGFR (Fig. 1.2) [3].

Preclinical studies have shown that both anti-EGFR antibodies and EGFR-TKIs block the *in vitro* growth of human NSCLC cell lines by inhibiting receptor phosphorylation and downstream protein phosphorylation, including MAPKs and Akt [3]. Moreover, the two classes of inhibitors have been assessed in combination with conventional cytotoxic chemotherapeutic drugs or radiation therapy. EGFR monoclonal antibodies have been shown to synergize with cisplatin or doxorubicin in NSCLC xenograft models. These antibodies also synergize with radiation and paclitaxel in both *in vitro* and *in vivo* studies. However, in the case of the small molecule inhibitors, neither

gefitinib nor erlotinib showed improved response rate or disease-free progression when used in combination with chemotherapeutic agents [24, 85].

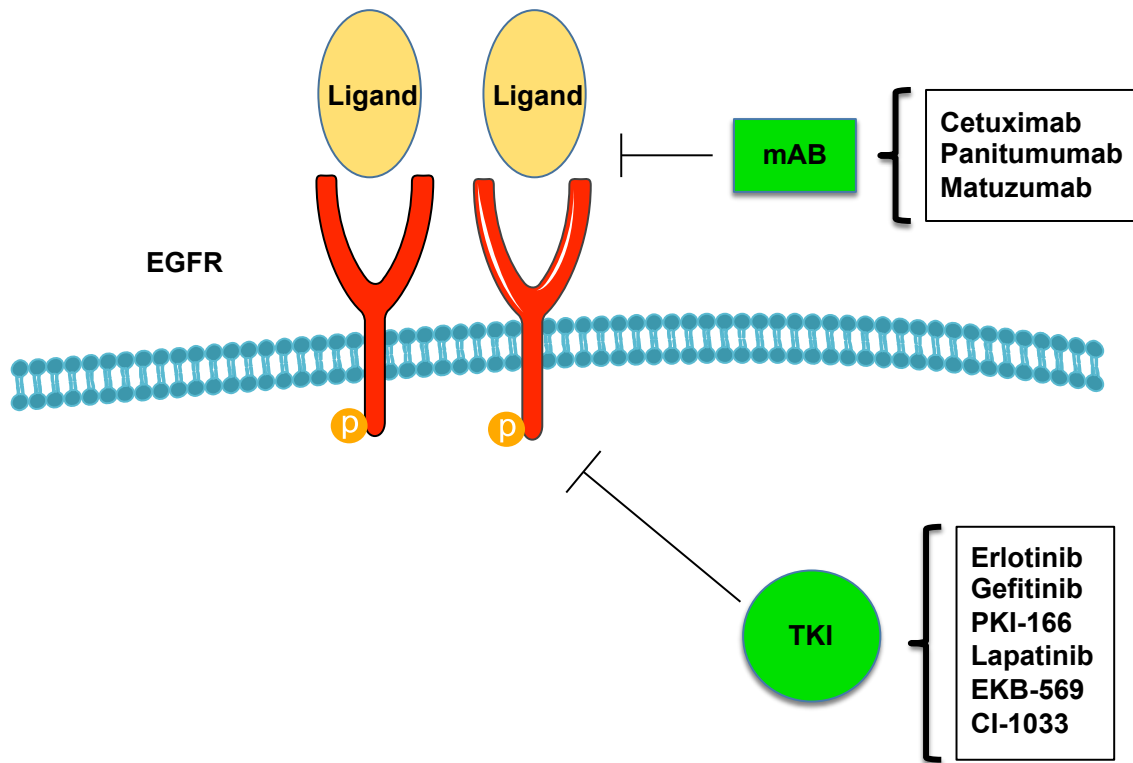


Figure 1.2 Antibodies and small molecules intercepting EGFR signaling

Anti-EGFR monoclonal antibodies and small molecule EGFR TKIs have been used in lung cancer treatment. mABs bind to the extracellular domain of EGFR and compete with endogenous ligands for receptor binding, and block ligand-induced receptor activation. The small molecule EGFR inhibitors compete with ATP to bind the catalytic domain of the kinase, which in turn inhibits EGFR autophosphorylation and downstream signaling.

Resistance to EGFR inhibitors in NSCLC

Despite the initial success of TKIs for the treatment of lung cancer patients harboring EGFR activating mutations, most patients develop resistance over time and progress to advanced stages of the disease. Several mechanisms have been reported for this acquired resistance, including secondary mutations in EGFR, parallel pathway activation, phenotypic transformation, and additional genetic alterations.

1. Presence of secondary mutations in EGFR

Secondary mutations in EGFR are documented in patients that develop resistance to EGFR TKIs. The most common is the T790M activating point mutation in exon 20 that substitutes the gatekeeper methionine for threonine, which interferes with the binding of reversible TKIs. The T790M substitution changes the affinity of the drug to the ATP pocket and enables ATP binding at the level of the wild-type EGFR. T790M is found in about 50 % of patients who developed acquired TKI resistance [94]. The use of second-generation TKIs such as afatinib and neratinib, which irreversibly bind to EGFR have been evaluated to overcome the effect of acquired resistance. Although the results from preclinical studies have been encouraging, the drugs did not increase response rate and progression free survival of lung cancer patients [43, 96, 158].

2. Parallel activation of downstream signaling pathways

Resistance to EGFR TKIs could also be acquired through mechanisms that involve activation of EGFR downstream targets such as Akt and ERK independent of EGFR. These mechanisms mainly involve amplifications of the MET oncogene and overexpression of its ligand, hepatocyte growth factor (HGF). These dysregulations

bypass inhibition of EGFR and render resistance to TKIs [44, 152]. MET is a tyrosine-kinase receptor implicated in cell motility, growth and angiogenesis in various tumors. Overexpression of HGF or MET amplification has been reported in close to 20 % of lung tumors exposed to EGFR TKIs. MET receptor activation results in EGFR-independent phosphorylation of ErbB3 and downstream activation of the PI3K/Akt pathway. This process allows the survival of cells with MET amplification in the presence of the EGFR TKI. MET amplification can occur either with or without EGFR T790M mutation. Although ~ 60 % of MET amplifications occur without the T790M mutation, studies have also reported a complementary role of the two dysregulations [14].

3. Phenotypic transformation - epithelial to mesenchymal transition

During embryonic development a balance between epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET) maintains proper cell morphology. Epithelial cells are polarized with tight connection to adjacent cells whereas mesenchymal cells exhibit a spindle-like morphology. In the context of cancer, EMT is marked by loss of epithelial cell junction proteins such as E-cadherin and gain of mesenchymal markers such as vimentin [191]. Mesenchymal type cells also possess enhanced motility and invasive phenotype, and the expression of genes associated with EMT has been correlated with poor patient prognosis in NSCLC [21, 209]. Studies have found that up-regulation of Notch-1 induces mesenchymal phenotype in lung cancer cells that are resistant to TKIs. Moreover, inhibition of Notch-1 signaling augmented sensitivity to TKI [210]. The aberrant expression of transforming growth factor (TGF- β) is also a potent inducer of EMT [160, 212]. Erlotinib-resistant NSCLC cells with

mesenchymal phenotype possess high levels of TGF- β expression. In addition, blockade of TGF- β signaling in combination with EGFR inhibition was shown to have synergistic effects on EGFR inhibition and cell viability [212].

4. Acquired genetic alterations other than EGFR mutations

PIK3CA mutations - Studies indicate that 5 % of lung cancers that developed acquired resistance to TKIs have PIK3CA mutations [159]. In several clinical trials, the combination of a pan-PI3K inhibitor with gefitinib resulted in enhanced efficacy in patients with acquired resistance to EGFR TKIs. However, many of the patients had toxicities, underscoring the challenge of blocking the two pathways that are critical for cell survival [51].

HER2 amplification: Mutations (approximately 2 %) and amplifications (2-5 %) of HER2, a member of the EGFR family, have been reported in patients with lung adenocarcinomas. Dysregulation of HER2 in lung cancer is associated with females, non-smokers, and Asian backgrounds. Additionally, 12 % of lung cancer patients exposed to TKI have amplification of the HER2 gene and present a more advanced stage of the disease. HER2 amplification was also shown to be mutually exclusive with EGFR T790M mutations [115].

ERK2 amplification: Approximately 5 % of tumors resistant to TKIs have amplification of ERK2. Furthermore, MEK inhibitors effectively restored the sensitivity of EGFR TKIs. Similar to HER2 amplification, ERK2 amplification is mutually exclusive with T790M mutation [179].

BRAF mutation: V600E and G469A, mutations in the BRAF, occur in 1 % of tumor samples that are resistant to TKIs. Although the V600E mutation appears in patients with the EGFR T790M mutations, the G469A mutation is mutually exclusive [130].

JAK2: JAK proteins phosphorylate signal transducer and activator of transcription (STAT), which forms a homodimer that translocates into the nucleus where it binds to DNA and promotes transcription of genes. Studies have shown that JAK2 activation induces resistance to EGFR TKIs and that JAK 2 inhibitors restore the sensitivity to erlotinib in cellular and xenograft models [2, 68].

1.2 Protein kinase C

Protein kinase C (PKC) belongs to the AGC superfamily of kinases. On the basis of structural, functional and biochemical properties, PKC have been classified into three subgroups; conventional, novel and atypical. The conventional (cPKCs α , β I, β II and γ) are diacylglycerol (DAG) and phorbol ester sensitive and respond to Ca^{2+} . The novel PKCs (nPKCs δ , ϵ , η and θ) only respond to phorbol esters or DAG. The atypical PKCs (aPKCs ι and ζ) are independent of DAG and Ca^{2+} . cPKCs and nPKCs are activated in response to an increase in DAG levels in the plasma membrane. This increase could be triggered by activation of receptor tyrosine-kinases, which couple to phospholipase C γ (PLC γ), or G-protein-coupled receptors (GPCRs) that couple with phospholipase C β (PLC β). PLCs in turn hydrolyze phosphoinositide 4,5-biphosphate (PIP₂) into second messengers inositol triphosphate (IP₃) and DAG. Binding of DAG (or phorbol esters) occurs in the C1 domains located in the regulatory region. Additionally, the cPKCs have a C2 domain that is required for Ca^{2+} binding. In the case of the atypical PKCs, regulation is achieved through their N-terminal PB1 domain. Although DAG is an important lipid second messenger for PKC activation, PKC-mediated responses could also occur through protein modifications [66].

At the membrane, PKCs undergo conformational changes that release the inhibition exerted by the pseudosubstrate sequence bound to the active site. In the open conformation, PKCs undergo a series of serine/threonine phosphorylations to achieve full activation. Phosphorylation in the activation loop at a TFCGT motif, conserved in many members of the AGC kinase superfamily, is required for full activation of PKCs. This

activation is catalyzed by phosphoinositide-dependent kinase 1 (PDK1) recruited by PIP3. Additional phosphorylations also occur at the C-terminal to lock the kinase domain in its active conformation [136]. At the plasma membrane, each PKC isozyme interacts with its anchoring protein, receptor of activated C-Kinase (RACK) and phosphorylates nearby substrates (Fig. 1.3) [121].

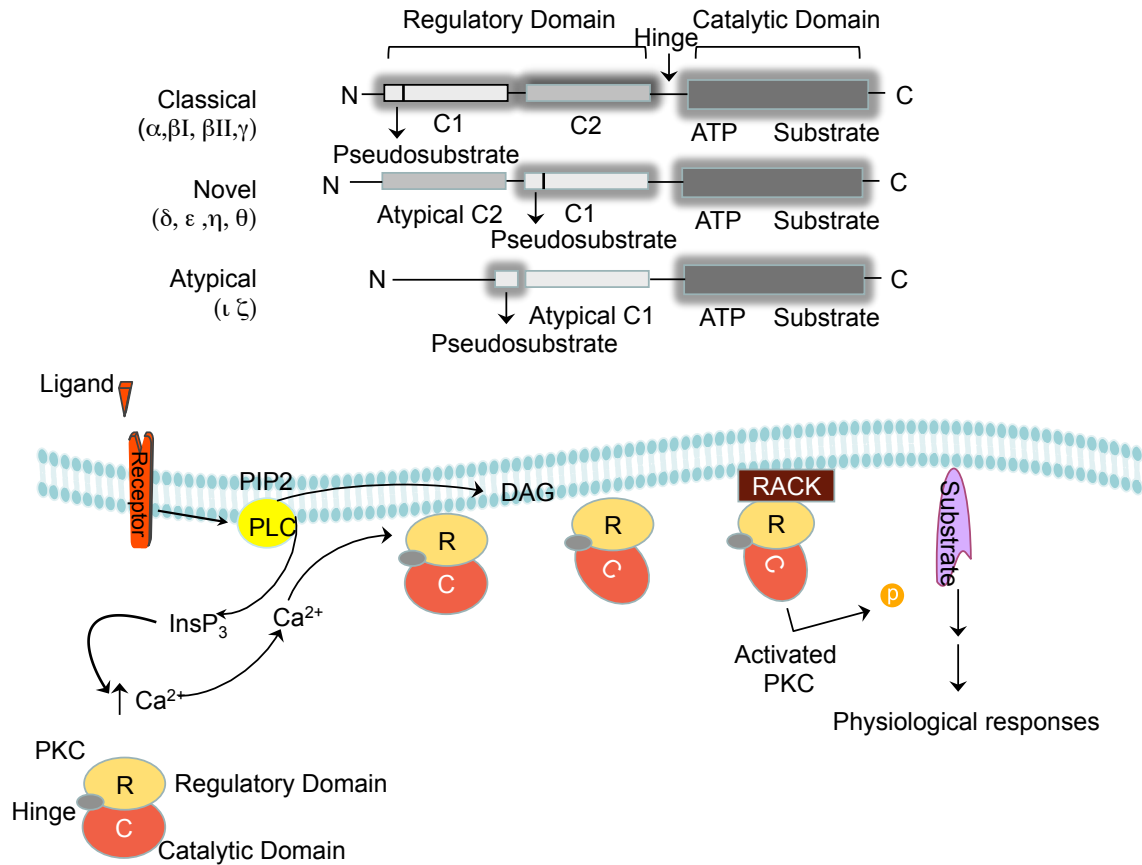


Figure 1.3 Structure and activation of PKC isozymes

cPKCs contain DAG responsive C1 domains and calcium binding C2 domains. The nPKC have C1 for the interaction with DAG but lack calcium binding C2 domains. Atypical PKCs are responsive neither to DAG nor to calcium. Upon increase in DAG (also calcium for cPKCs), PKCs localize to the plasma membrane where they interact with their specific anchoring protein, receptor for activated C-kinase (RACK) and phosphorylate nearby substrates leading to various cellular responses.

Protein kinase Cs in cancer development and progression

PKC isozymes play important roles in the development and progression of many cancers by regulating cell cycle, survival, apoptosis, cell motility and malignant transformation. Although mutations and chromosomal rearrangements of PKC isozymes are rare events in cancer, altered expressions of PKCs have been associated with the development of many cancers including lung cancer (Table 1.2). PKC α , PKC β , PKC δ , PKC ϵ and PKC ι have been implicated in lung cancer malignancy and the following section focuses on their roles in cancer progression.

	Alpha	Beta	Delta	Epsilon	Iota
Breast	Assender, JW et al 2007 ↑; Kerfoot, C et al 2004 ↓	Gokmen-Polar, Y et al 2010 ↑		Pan, Q et al 2005 ↑	Kojima, Y et al. 2008 ↑
Colon	Suga, K et al 1998 ↓				Murray, NR et al. 2004 ↑
Lung				Bae, KM et al 2007 ↑	Regala, RP et al. 2005b ↑
Prostate		Metzger, E et al 2010 ↑	Kharait, S et al 2006 ↑; Villar et al 2007 ↑	Aziz, et al 2007 ↑; Koren, R et al , 2004 ↑; McJilton, MA et al, 2003 ↑	Ishiguro, H et al. 2009 ↑

Table 1.2 Altered expressions of PKC isozymes in primary tumor samples

PKC α : This PKC plays important roles in many cellular processes such as cell proliferation, cell cycle, apoptosis, and motility, and regulates the expression of genes associated with multidrug resistance. PKC α can act as a tumor suppressor or tumor promoter in different contexts. PKC α has been described as a pro-survival kinase in breast, endometrial, glioma and androgen-independent prostate cancer cells. These studies indicate that PKC α mediates mitogenic signals through the activation of the MAPK pathway, induction of CDK1 and inhibition of p21 [128, 171]. In contrast, PKC α is widely reported as a tumor suppressor in intestine and skin tumors. Analysis of tissue samples from PKC $\alpha^{-/-}$ mice indicates higher expression of inhibitor of DNA binding (Id1) known for its tumorigenic properties in the crypts of the small intestine and colon tumors [141]. In skin cancer, PKC α overexpression suppresses tumor formation induced by the carcinogen DMBA. Similarly, PKC α has been shown to act as a tumor suppressor in KRAS-driven lung cancer models [73], and activation of PKC α in H358, H441 and H322 NSCLC cells in late G1/early S phase leads to senescence through up-regulation of p21^{cip1} [133]. In contrast, a PKC α antisense oligonucleotide (ODN) exhibited additive antitumor effects on lung carcinomas, and depletion of PKC α in human lung carcinoma LTEP cells, significantly reduced tumor formation and size in nude mice underscoring the divergent role of PKC α in lung cancer cells [196].

PKC α is ubiquitously expressed in human tissues, but exhibits altered expression in various cancer tissue samples [90]. A study on the role of PKC α in bladder cancer reported that tumor specimens have elevated levels of PKC α compared to normal bladder

tissue. Moreover, the levels of PKC α correlate with the malignant grade of the tumor samples [4]. There are controversial reports regarding the relationship between PKC α expression and advanced stages of breast cancer. Several clinical studies correlated overexpression of PKC α with advanced breast carcinoma and poor clinical outcomes of endocrine therapy. Tumor samples that have high staining for PKC α are negative for estrogen receptor expression. On the other hand, a clinical study involving 46 advanced stage breast cancer patients showed low PKC α staining in tumors compared to normal tissue [90]. In endometrial tumors, PKC α staining was variable among patients and different sections of tumors. Nevertheless, overall endometrial tumors express high PKC α levels relative to normal tissue [70]. Analysis of 30 tissue samples derived from human colorectal cancer patients indicated low expression of PKC α in tumor specimens [172]. Likewise, malignant renal cell carcinoma (RCC) tumors have much lower PKC α staining than benign oncocytomas [194] .

By far the most consistent function of PKC α is its role in cell motility in different cancer types. PKC α has been implicated in migration of NSCLC cells in response to cytokines [31]. ErbB2-dependent activation of PKC α promotes cell invasion in breast cancer cells [183]. In glioma, the phorbol ester PMA enhances invasion and migration through activation of PKC α [105]. Activation of PKC α in response to various stimuli also induces invasion and migration in hepatocellular, melanoma, ovarian and endometrial cancer cells [22, 112, 197, 208].

PKC β : PKC β I and PKC β II are the two splice variant of PKC β derived from the same gene. PKC β is mainly implicated in inflammation and angiogenesis but also plays roles in cell cycle, proliferation, differentiation and metastasis. The role of PKC β is well reported in prostate, breast and colorectal cancers, as well as in lymphomas and glioblastomas.

PKC β I is highly expressed in prostate cancer tumors compared to benign prostatic hyperplasias [116]. In the case of PKC β II, high levels are localized in endothelial cells leading to increased angiogenic capacity of prostate cancer cells. Furthermore, PKC β II controls cell proliferation and tumor growth. *In vivo*, tumor formation of PC3 cells was attenuated using a PKC β II specific inhibitor [64]. PKC β II protects chronic lymphocytic leukemia cells from apoptosis by regulating Bcl2 and Bim proteins. Phosphorylation of Bcl2 by PKC β leads to inhibition of apoptosis by sequestering Bim. Moreover, high stromal PKC β II was found in patients with various hematological malignancies, which correlates with more aggressive stages of the disease. PKC β is shown to regulate the activation of stromal NF- κ B that results in the survival of leukemic B cells. *In vivo*, crossing T cell leukemia (TCL) transgenic mice that develop leukemia with PKC β deficient mice abrogated the development of chronic lymphocytic leukemia (CLL) [111].

In colon and breast carcinomas, the two splice variants have opposite roles. PKC β II enhances proliferation of intestinal epithelium and is required for the initiation of colon cancer [168]. Transgenic mice that overexpress PKC β II develop preneoplastic lesions and become susceptible to azoxymethane-induced colon carcinogenesis. The levels of colonic β -catenin were also elevated, accounting for proliferation due to

activation of the PKC β /Wnt/APC/ β -catenin pathway [125]. Furthermore, PKC β II promotes cell invasion through mechanisms that involve the RAS/MAPK and Rac1 pathways. Spindler *et al.* reported that 18 % of primary colon adenocarcinomas exhibit very high levels of PKC β II, and high expression of PKC β II correlates with poor survival rates [168]. On the contrary, overexpression of PKC β I suppresses tumor formation of HT29 and SW480 colon cancer cells in xenograft models [61]. In breast cancer, overexpression of PKC β I suppresses proliferation, tumorigenic capacity, and metastasis while high levels of PKC β II are reported in several breast cancer cell lines and patient samples [101]. In murine cell lines derived from mammary tumors, overexpression of PKC β I leads to increased latency and slow growth of tumors in mice. Moreover, tumor cells that overexpress PKC β I have attenuated metastatic capacity to the lungs. On the other hand, PKC β II regulates VEGF-induced angiogenesis and growth in breast cancer cells. The expression levels of cytoplasmic PKC β II positively correlate with HER2 levels while nuclear PKC β II correlates positively with ER levels [60].

PKC β reduces apoptotic signals and cell invasion in gastric and hepatocellular carcinomas. In hepatocellular carcinomas, PKC β II-mediated ERK/heat shock protein 27 (HSP 27) activation is responsible for cell motility and invasion. Down-regulation of PKC β I expression and activity by a COX-2 specific inhibitor (SC-236) attenuates apoptotic response of gastric cancer cells [216]. Much of the studies on glioblastomas and lung cancer cells were done with the PKC β specific inhibitor enzastaurin. In glioblastomas, PKC β regulates proliferation by suppressing the activity of GSK3, S6

kinase and Akt. Moreover, the effect of the PKC β inhibitor was shown using U87MG human glioblastoma cells inoculated in nude mice [185]. In NSCLC, treatment with enzastaurin inhibits the transcription of genes associated with tumor progression such as u-PAR and VEGF-C and up-regulates tumor suppressors. PKC β inhibition in NSCLC cells also resulted in reduced migration, invasion and metastasis [204].

PKC δ : PKC δ functions as a tumor suppressor as well as a pro-survival kinase in several cancer cell types. PKC δ activation depends on multiple factors such as proteolytic activation, tyrosine phosphorylation, intracellular localization, and the nature of apoptotic stimuli [11]. Studies have shown that activation of PKC δ inhibits cell cycle progression and down-regulation of PKC δ facilitates tumor formation. In lung cancer cells, overexpression of PKC δ led to PMA induced G1 arrest by regulating the expression of cell cycle inhibitor p21^{cipl} [127, 156]. Suppression of p53 due to an increase in PKC δ expression was associated with colon cancer progression. The function of PKC δ as a pro-apoptotic protein was further established by the observation that treatment with genotoxic agents results in cleavage of PKC δ by caspase-3 [149]. In androgen-dependent prostate cancer cells, PKC δ activation by PMA triggers the extrinsic apoptotic cascade through the activation of an autocrine loop that involves the secretion of the apoptotic factors TNF α and TRAIL. This pro-apoptotic cascade can be blocked with pharmacological agents against p38 MAPK and JNK, suggesting a role for MAPK cascade as effectors of the apoptotic response mediated by PKC δ [63]. Contrary to its role as a pro-apoptotic kinase, studies in breast, lung, pancreatic and liver tumor models have linked PKC δ to survival and enhanced resistance to anti-cancer drugs [11]. PKC δ specific inhibitors as

well as kinase-dead mutants were shown to attenuate cell survival in NSCLC and breast cancer cells. Moreover, PKC δ expression was found to be higher in estrogen receptor-positive human breast tumors. Ectopic expression of PKC δ also enhanced anchorage-independent growth and resistance to cytotoxic drugs in pancreatic and liver cancer cells [120]. Studies using genetically engineered mouse models also contribute to unravel roles of PKC δ in tumorigenesis. For example, PKC δ skin transgenic mice are resistant to tumor promotion by DMBA and PMA [145]. In lung cancer, KRAS-driven lung tumorigenesis was abrogated in PKC δ knockout mice [176]. Similarly, ErbB2 overexpressing mice in a PKC δ -null background delays breast tumorigenesis [1].

PKC δ is generally reported as a positive regulator of cell motility. In pancreatic and renal cell carcinoma, PKC δ phosphorylation led to enhanced cell motility [45, 120]. In glioma cells, PKC δ increased MMP-12 expression, which results in increased invasion [157]. Overexpression of claudin-1 activates a c-Abl-PKC δ signaling pathway, leading to a marked elevation in MMP-2 activity and invasion in human liver cancer cells [214]. The chemokine CCL5 induces the production of MMP-9 and an invasive phenotype in oral cancer cells also via a PKC δ -dependent mechanism [34]. In prostate cancer cells, PKC δ activation induces migration downstream of EGFR. Moreover, overexpression of PKC δ in prostate cancer cells enhances collagen secretion [193]. In gastric carcinoma cells, TGF- β -induced PKC δ expression enhances cell spreading, motility and invasion [32].

PKC ϵ : This nPKC is overexpressed in many cancer types including skin, breast, lung, prostate, renal and head and neck squamous cell carcinoma. Overexpression of PKC ϵ in

these cancers is associated with the development and progression of the disease. High PKC ϵ staining in primary breast tumors correlates with a high histological grade, positive HER2 receptor status, and loss of hormone receptor [12]. Overexpression of PKC ϵ has also been reported in greater than 90 % of NSCLC and prostate tumors [7].

PKC ϵ functions as an anti-apoptotic kinase through its regulation of the cell cycle and expression of genes associated with cell survival. Studies have shown that PKC ϵ promotes cyclin D1 induction leading to G1 to S phase progression during the cell cycle. Moreover, PKC ϵ has been implicated in mitogenic signals through mechanisms that involve caspases, Bcl2 family members, the Raf/MEK/ERK pathway and other pro-survival molecules [25, 26]. In NSCLC, PKC ϵ protects cells against TRAIL-induced apoptosis and depletion of PKC ϵ leads to enhanced expression of pro-apoptotic proteins. PKC ϵ inhibition also attenuates tumor growth and metastatic dissemination in nude mice [25, 47]. In prostate cancer, PKC ϵ depletion impairs the activation of the NF- κ B pathway in response to TNF α . Moreover, overexpression of PKC ϵ in normal immortalized prostate epithelial cells causes ERK and Akt activation and confers growth advantage. Prostate-specific overexpression of PKC ϵ in mice led to the formation of preneoplastic lesions [18, 54]. Additionally, in androgen-dependent LNCaP cells, overexpression of PKC ϵ results in tumor growth in nude mice. Similarly, overexpression of PKC ϵ enhances tumorigenic and metastatic potential of breast cancer cells [108]. The role of PKC ϵ in skin cancer initiation and progression has been shown using a skin-specific PKC ϵ overexpressing transgenic mouse model. These mice exhibit phenotypic abnormalities

including inflammation, hyperkeratosis and ulceration resulting in a highly malignant and metastatic squamous skin cell carcinoma [203].

PKC ϵ : This aPKC is an oncogene required for maintenance of the transformed phenotype of NSCLC cells with KRAS mutations. Moreover, the PKC ϵ -Par6-Rac1 signaling was found to be important for the transformation of NSCLC cells [50, 146]. PKC ϵ also regulates the Rac1/Pak1/MEK/ERK signaling pathway involved in cell proliferation of NSCLC cells [147, 217]. Gene expression microarray analysis of U87MG glioma cells showed that PKC ϵ suppressed the expression of RhoB, which has previously been reported to have a role in actin stress fiber formation [8]. *In vitro* and *in vivo* experiments also revealed the involvement of PKC ϵ in prostate cancer cell growth through secretion of IL-6. Indeed, PKC ϵ activates transcription of the IL-6 gene through NF- κ B and AP-1, thus arguing for its involvement in hormone-independent prostate cancer cells [82]. PKC ϵ is also a potential oncogene in ovarian cancer, as enhanced PKC ϵ protein levels are associated with increased cyclin E protein expression and proliferation in ovarian cancers [217].

1.3 Nuclear factor- κ B (NF- κ B) transcription factors

Nuclear factor- κ B (NF- κ B) represents a family of structurally related transcription factors that are implicated in various cellular processes including inflammation, proliferation, angiogenesis, transformation, apoptosis, invasion, and resistance to chemotherapy and radiation therapies. There are five members of the mammalian NF- κ B family; RelA (p65), RelB, c-Rel, NF- κ B1 (p50/p105) and NF- κ B2 (p52/p100). Each member contains a highly conserved 300 amino acid N-terminal region called Rel homology domain (RHD). This region comprises sub-domains for dimerization, nuclear localization (nuclear localization sequence, NLS) and DNA binding. The five members are further subdivided into two groups based on their catalytic activity. RelA, RelB and c-Rel contain C-terminal transactivation domains, which are made up of serine and hydrophobic amino acids important for the transactivational activity. The RHD of RelA and c-Rel contains a phosphorylation site for Protein Kinase A (PKA) that is important for transcriptional activity. In the case of the NF- κ B proteins p100 and p105, the C-terminal region consists of multiple copies of ankyrin repeats, which keeps the proteins in their inactive form. The NF- κ B proteins are converted to active DNA-binding proteins (p105 to p50, and p100 to p52) by proteolytic cleavage or arrested translation, and as they lack the transactivation domain, they cannot act as activators of transcription except when they form dimers with the Rel proteins. NF- κ B family members form multiple homo-and heterodimers depending on the cell type, nature of the stimulus, and the length of exposure. In general, p50/RelA or p52/RelA complexes are the most common dimers [13, 59].

NF- κ B activation pathways

Activation of NF- κ B occurs primarily by two signaling pathways; canonical and non-canonical pathways. Activation of the I κ B kinase (IKK) complex is the common upstream regulatory step in both pathways of NF- κ B activation. The IKK complex includes catalytic kinase subunits IKK α and IKK β , and a regulatory subunit called NF- κ B essential modulator (NEMO). Although the signals mediated by IKK α and IKK β differ, both could phosphorylate I κ B and exhibit similar structural organization, including kinase domain, leucine zipper and helix-loop-helix domains with 52 % amino acid homology. IKKs become activated via phosphorylation of serine residues in the kinase domain (residues 176 and 180 in the case of IKK α and residues 177 and 181 in the case of IKK β) by IKK kinases. The canonical pathway is activated in response to multiple stimuli, such as ligand binding to TNF receptors, IL-1 receptors, T-cell receptors, B-cell receptors, and Toll-like receptor 4 (TLR4). This pathway induces the transcription of genes implicated in inflammatory response, cell motility and growth [29, 59, 80].

Association of NF- κ B dimers with their inhibitory proteins, inhibitors of NF- κ B (I κ B), sequesters the complex in the cytoplasm during the inactive state. The human I κ B family of inhibitory proteins includes I κ B α , I κ B β , I κ B γ , and I κ B ϵ , which have different affinities for individual NF- κ B dimers. I κ Bs possess several ankyrin repeats, which are 33 amino acid sequences that mediate binding to NF- κ B dimers. Unprocessed NF- κ B1 (p105) and NF- κ B2 (p100) also function as I κ B proteins. I κ B proteins sequester the NF- κ B dimers in the cytoplasm by hiding their nuclear localization sequences. With the

exception of I κ B β , the expression of I κ B proteins is regulated by NF- κ B resulting in an autoregulatory loop of NF- κ B signaling. The NF- κ B/I κ B α complex is activated by phosphorylation on conserved serine residues in the N-terminal portion of I κ B. This modification occurs at ser-32 and ser-36. Phosphorylation targets I κ B α for ubiquitination by the SCF-ubiquitin ligase complex, which leads to the degradation of the inhibitory subunit by the 26S proteasome. This process enables the p50/p65 dimer to translocate to the nucleus and bind to its DNA-binding site in the promoter or enhancer regions of specific genes. Several modifications of the p65 protein occur during active transcription of target genes. Phosphorylation of the p65 subunit by PKA or MAPKs at ser-276 is important for the recruitment of cAMP response element-binding (CREB) protein/p300 [59]. Additionally, phosphorylation by casein kinase II and IKKs increases the transcriptional activity of NF- κ B. Epigenetic modifications such as acetylation, methylation and demethylation of p65 also play a key role in the regulation of NF- κ B transcriptional activity [28].

In the case of the non-canonical pathway, activation of NF- κ B is triggered by ligand binding to lymphotoxin β receptor, B-cell-activating factor, CD40, and CD30. Activation of the non-canonical pathway is involved in the development of lymphoid organs and the adaptive immune system. This pathway depends on the recruitment of TNF associated factor (TRAF) to the membrane and activation of IKK α by NF- κ B inducing kinase (NIK). Activated IKK α forms a homodimer and phosphorylates p100, leading to its ubiquitination and generation of the p52 protein. p52 forms a heterodimer with RelB and localizes to the nucleus [59]. A third pathway that does not involve IKKs

has also been described for NF- κ B activation. This atypical pathway includes DNA damage followed by activation of casein kinase 2 downstream of p38, resulting in phosphorylation and degradation of I κ B (Figure 1.4) [175].

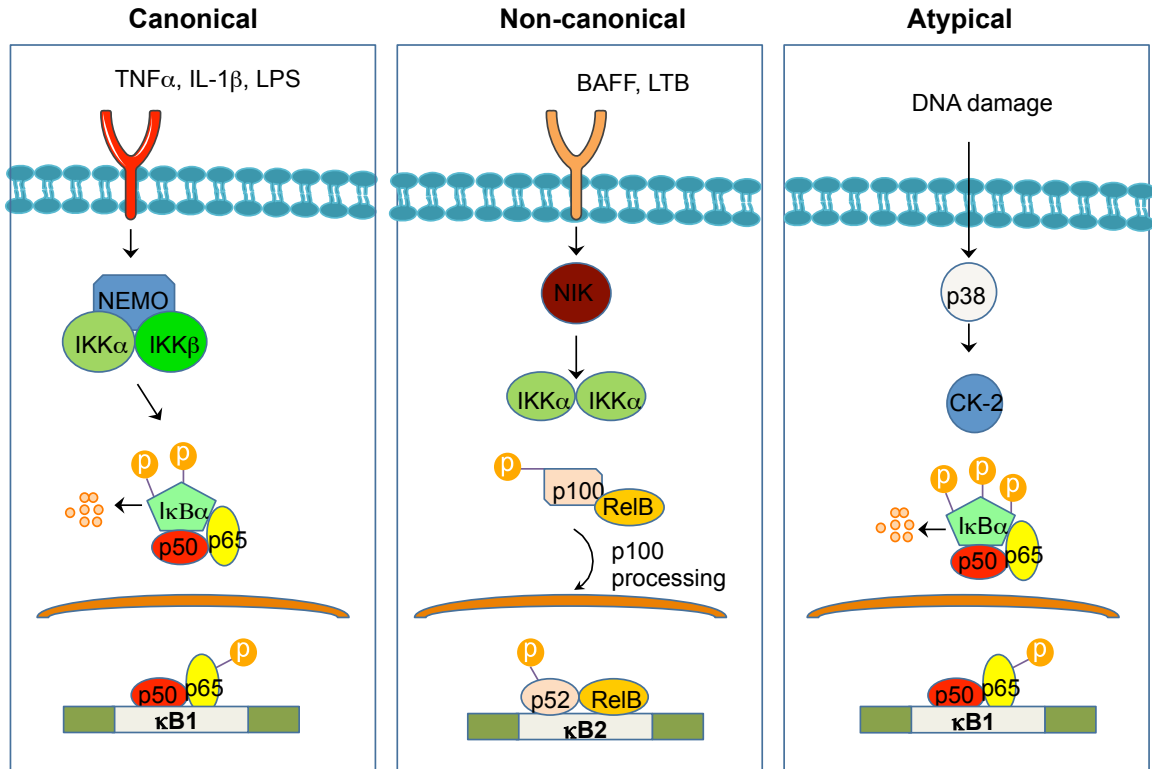


Figure 1.4 NF- κ B activation pathways

In the canonical pathway, $\text{TNF}\alpha$, $\text{IL-1}\beta$ or LPS binds to their respective receptors and lead to IKK-mediated activation of I κ B α . I κ B α is degraded following activation and release p50/p65 to localize to the nucleus. In the non-canonical pathway, lymphotoxin β ($\text{LT}\beta$) or B-cell activating factor (BAFF) are implicated in NF- κ B inducing kinase (NIK)-mediated activation of IKK α . IKK α then phosphorylates p100, which then undergoes proteasomal processing to generate p52. RelB/p52 dimers are then released and translocate to the nucleus. The atypical pathway is triggered by DNA damage, which activates p38-induced casein kinase-2 (CK-2) and results in phosphorylation of I κ B α .

NF- κ B and cancer

Dysregulation of the NF- κ B signaling pathway is a common feature of many human diseases including cancer. NF- κ B is important in integrating multiple stress stimuli and regulating innate and adaptive immune responses seen in inflammation, which is associated with or precede cancer initiation [15]. NF- κ B activation occurs in premalignant cells that are destined to undergo malignant conversion as well as in cells that are recruited to the tumor microenvironment. These cells produce cytokines, angiogenic factors, and proteases that degrade the extracellular matrix to support cancer development and progression. The microenvironment cells include macrophages, dendritic cells, neutrophils, mast cells, T cells and B cells [13]. Generation of several inflammatory cytokines, including TNF α , IL-1 β and IL-6, in some of these cells is known to be dependent on the canonical NF- κ B pathway activation induced by inflammation. Secreted TNF α and IL-1 β act on the premalignant cells to activate NF- κ B, which induces expression of genes involved in inhibition of apoptosis and promotion of proliferation and angiogenesis. NF- κ B activation in the microenvironment cells could also promote production of reactive oxygen species (ROS), which induce DNA damage of the premalignant cells. Such DNA damage activates NF- κ B, which induces expression of anti-apoptotic genes to promote cancer progression [29, 59].

Various studies identified the role of NF- κ B in both hematological and solid tumors. A viral homolog of c-Rel was shown to cause aggressive lymphomas and leukemias in chickens [27]. A wide variety of haematopoietic proliferations show dysregulated NF- κ B signaling pathway including mutations in I κ B α , amplification of c-

Rel, and chromosomal rearrangements in the *NF-κB2* gene. In solid tumors, mutations of the NF-κB signaling pathway have been documented in breast and prostate cancers [178]. Studies have shown mutations in the NF-κB1, IKKβ as well as IκBα and IκBε as well as high levels of c-Rel in breast cancer [144]. A transgenic mouse that overexpressed c-Rel in the mammary gland developed one or more mammary tumors and had increased expression of NF-κB target genes, such as cyclin D1, c-myc and Bcl-XL [17]. In prostate cancer, gene fusion between IKKβ and transpootin 1 resulted in elevated IKKβ expression [138]. Although RelA, RelB and NF-κB1 mutations are rare in other human cancers, these subunits are constitutively activated in a wide variety of human tumors. Increased RelA nuclear localization or activity is present in many tumors and is associated with tumor progression [74]. Gastric carcinoma shows increased nuclear translocation of RelA in comparison with adjacent normal epithelial cells and correlates with tumor invasion. NF-κB activation has also been identified in squamous cell carcinomas of the head and neck. Inhibition of NF-κB activity in these tumors attenuates cell survival and tumor growth[190]. The expression of IKKα has been correlated with increased expression of NF-κB and COX-2 in colorectal carcinomas. *In vitro* studies have also suggested a positive role for NF-κB in cell transformation in prostate and colon epithelial cells, fibroblasts, and lymphocytes [55, 195]. In lung cancer, tumor samples showed high levels of NF-κB activation associated with advanced stage of disease and poor prognosis. NF-κB activation in lung cancer has also been correlated with loss of p53 function and KRAS mutation and mediates secretion of inflammatory cytokines that play an important role in lung cancer development [118].

PKC and NF- κ B in cancer

The PKC family has long been known to play an important role in cell growth and differentiation by regulating the activity of transcription factors such as NF- κ B. Phorbol esters can induce transient activation of NF- κ B and control the expression of MMPs [75]. It has also been shown S6 kinase is responsible for phosphorylation of I κ B α at Ser-32 after PMA stimulation [135]. Initial studies that link PKCs to NF- κ B activation in cancer were reported for atypical PKCs. PKC ζ phosphorylates and inactivates NF- κ B upstream kinase, IKK β at ser-177 and ser-181 in response to TNF α . Additionally the loss of PKC ζ was shown to impair RelA phosphorylation and inhibit the translocation of NF- κ B to the nucleus. Deletion of PKC ζ reduces NF- κ B activation in lung in response to both TNF α and IL-1 β [40, 124]. The other atypical PKC isozyme, PKC ι also mediates phosphorylation of IKK β in response to TNF α and induces NF- κ B dependent transcription of the IL-6 in prostate cancer. Recently, our laboratory identified a key role of PKC ϵ in TNF α dependent activation of NF- κ B in prostate cancer. PKC ϵ was shown to be a part of the TNFRI signaling complex that leads to NF- κ B activation [54]. Yet another novel PKC isozyme, PKC δ was reported to facilitate RelA binding to the promoters of target genes in the nucleus [110]. Inhibition of both PKC δ and PKC ϵ has been shown to prevent TNF- α -induced NF- κ B activation, determined by NF- κ B binding activity and I κ B α degradation in pancreatic cancer cells. Activation of the NF- κ B pathway is also attributed, in part, to alterations in classical PKCs. PKC β dependent

activation of NF- κ B, through modulation of the IKK complex, was found to be crucial in the survival of chronic lymphocytic leukemia [111, 123]. For PKC α , the mechanism for its interactions with NF- κ B was first shown using *in vitro* binding assays. PKC α forms a complex with NF- κ B upstream kinase, IKK. Additionally PKC α have been shown to interact with IKK β in response to PMA in 293T cells [135]. Mechanism for PKC α dependent NF- κ B activation was also extended to phosphorylation of RelA in glioma cells. In these cells, PKC α mediated activation of NF- κ B results in pro-survival phenotype [126].

Overall goals and significance of research

The goal of my thesis research has been to identify the association between PKC isozymes and different processes that contribute to lung cancer malignancy. Specifically, I focused on three different aspects: drug resistance, inflammation and tumorigenesis.

First, I aimed to determine whether PKCs modulate erlotinib resistance in NSCLC. I used parental and derived erlotinib-resistant cell lines to profile PKC isozyme expression and study their roles in altering drug sensitivity and mesenchymal phenotype. The outcome of my study established PKC α and PKC δ as important regulator of erlotinib sensitivity. Moreover, high expression of PKC α was found to be important for the maintenance of the mesenchymal phenotype. Second, I wished to identify the requirement of PKCs for interleukin mediated NF- κ B activation. I was able to show that activation of NF- κ B in response to IL-1 β is dependent on PKC α . These studies will contribute to establish PKC α specific inhibitors as combinational therapeutic options with indications of TKI resistance and NF- κ B hyperactivation.

Finally, based on previous findings from our lab implicating PKC ϵ signaling to lung cancer progression, I aimed to identify whether its function also extends to tumorigenesis using animal models. I crossbred KRAS mutant mice with PKC ϵ knockout mice, to study if PKC ϵ modulates oncogenic driven tumor formation. In addition, I aimed to generate an inducible PKC ϵ transgenic mouse model that could be used to study whether PKC ϵ could by itself drive lung tumor formation.

CHAPTER 2: PKC α mediates erlotinib resistance in NSCLC cells

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Abstract

Overexpression and mutational activation of the epidermal growth factor receptor (EGFR) plays an important role in the pathogenesis of non-small-cell lung cancer (NSCLC). EGFR tyrosine kinase inhibitors (TKIs) are given as a primary therapy for patients with EGFR activating mutations; however, the majority of these patients eventually develop resistance to TKIs. To address a potential role of PKC isozymes in the resistance to TKIs we used an isogenic model: the NSCLC H1650 cell line and its erlotinib-resistance derivative H1650-M3. H1650-M3 cells display a mesenchymal-like morphology driven by TGF β signaling [212]. We found that H1650-M3 cells display remarkably high levels of PKC α and reduced levels of PKC δ . Notably, RNAi depletion of PKC α from H1650-M3 cells caused a significant reduction in the expression of EMT markers vimentin, Zeb2, Snail and Twist. Moreover, pharmacological inhibition or silencing of PKC α sensitized H1650-M3 cells to erlotinib. Whereas ectopic overexpression of PKC α in parental H1650 cells did not alter the expression of EMT genes or confers resistance to erlotinib, it resulted in down-regulation of PKC δ expression. Forced PKC δ overexpression in H1650-M3 cells also sensitizes the resistant cells to erlotinib. In addition, we observed that treatment of H1650-M3 cells with the TGF- β inhibitor LY2109761 attenuates the expression of PKC α . Moreover, PKC α overexpression cooperates with TGF- β for the induction of specific EMT markers. Our results highlight a potential role for PKCs in EMT and erlotinib resistance in lung cancer cells, and suggest a potential therapeutic role for PKC α inhibition in lung cancer treatment.

2.1 Introduction

Lung cancer remains one of the major causes of mortality worldwide, accounting for more deaths than any other cancer cause. Diagnosis of lung cancer normally occurs in late stages of the disease process, thus limiting the options for treatment. The most common type of lung cancer (~85 %) is non-small cell lung cancer (NSCLC), which includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma [24, 162]. Genetic alterations in NSCLC tumors have been extensively studied, and include primarily oncogenic mutations in epidermal growth factor receptor (EGFR) and KRAS, as well as inactivation of tumor suppressor genes such as p53, PTEN, Rb, and p16 [77, 86, 148]. Mutations in EGFR, particularly deletion of exon 19 and L858R mutation in exon 21 occur in 10-50% NSCLC patients depending on the population studied [36, 56]. Small molecule tyrosine kinase inhibitors (TKIs) that reversibly inhibit EGFR at the ATP pocket domain, such as erlotinib and gefitinib, currently represent the first-line therapeutic option for EGFR-mutated NSCLC patients [3]. Although these therapies are initially efficacious, ultimately most patients develop resistance. Whereas resistance has been attributed in some cases to the acquisition of mutations in EGFR (such as the T790M mutation) or MET amplification [44, 94], the mechanisms behind the resistance to TKIs have not been fully evaluated. A deep understanding of the signaling mechanisms driving resistance is crucial for designing combinational therapy regimes to overcome this hurdle and extend life expectancy of NSCLC patients.

Protein kinase C (PKC) represents group of serine-threonine kinases involved in a variety of cellular functions, including mitogenesis, survival, and motility. This family of

kinases is composed of 11 members classified into three classes based on their biochemical and structural properties: calcium-dependent or conventional PKCs (cPKC α , β I, β II, and γ), calcium-independent or novel PKCs (nPKC δ , ϵ , η , and θ), and phorbol ester/diacylglycerol (DAG) unresponsive or atypical PKCs (aPKCs ζ and ι/λ) [66]. Decades of research have established key roles for different members of the PKC family in the progression of cancer [53, 66]. Individual PKC isozymes can either act as tumor promoters or tumor suppressors in various cancers including lung cancer. For example, PKC β has been proposed to be involved in lung tumorigenesis, and the PKC β inhibitor enzastaurin has been studied as a potential anti-cancer agent for lung cancer patients [186, 204]. Our laboratory recently showed that PKC ϵ , a kinase implicated in cell cycle progression and motility of NSCLC cells, is required for the tumorigenic and metastatic activities of NSCLC cells [25, 26]. On the other hand, PKC α and PKC δ negatively modulate NSCLC cell cycle progression. Most recently, Hill *et al.* provided direct evidence for a tumor suppressive role of PKC α in KRAS driven tumorigenesis [73]. Other reports on the contrary indicate that PKC α promotes NSCLC cell migration [31] arguing for divergent roles for this kinase in lung cancer progression. Likewise, diverse roles for PKC α and other members of the PKC family have been established in cell survival in NSCLC and other cancer types.

Towards the goal of determining a potential role of PKC isozymes in TKI resistance in lung cancer, here we took advantage of an isogenic NSCLC cell model of erlotinib-resistance generated by culturing the parental H1650 cell line in the presence of a high concentration of the TKI. Erlotinib-resistant cells were shown to display features

of epithelial-to-mesenchymal transition (EMT), a phenotype that was found to be maintained by the transforming growth factor beta (TGF- β) pathway [212]. Our study identified discrete roles for PKC isozymes in erlotinib resistance and EMT in NSCLC cells.

2.2 Results

Erlotinib-resistant cells display altered expression of PKC isozymes

Changes in the expression levels of PKC isozymes have been associated with the progression of many types of cancers, including lung cancer [53]. In order to determine a potential involvement of PKC isozymes in erlotinib resistance, we took advantage of an isogenic NSCLC cell model; the parental H1650 cell line and its erlotinib-resistant derivative H1650-M3 [212]. H1650 cells express the conventional PKC α , novel PKC δ and PKC ϵ , and atypical PKCs. Western blot analysis shown in Fig. 2.1A revealed a remarkable up-regulation of PKC α in the erlotinib-resistant variant H1650-M3. H1650-M3 cells also have reduced PKC δ levels. Determination of mRNA levels for these two PKCs led to similar conclusions. Indeed, H1650-M3 cells have 25-fold higher PKC α mRNA levels than the parental H1650 cells, whereas PKC δ mRNA levels are reduced by 5-fold in the erlotinib-resistant cell line (Fig. 2.1B).

PKC α is required but not sufficient to induce erlotinib resistance

To assess a potential association between altered PKC α expression and erlotinib-resistance, we used both pharmacological and RNAi approaches. As PKC α has been implicated in drug resistance in some cancer types [30, 99, 220] and its levels are strikingly high in erlotinib-resistant cells, we speculated that this PKC is involved in acquired resistance to erlotinib in NSCLC cells.

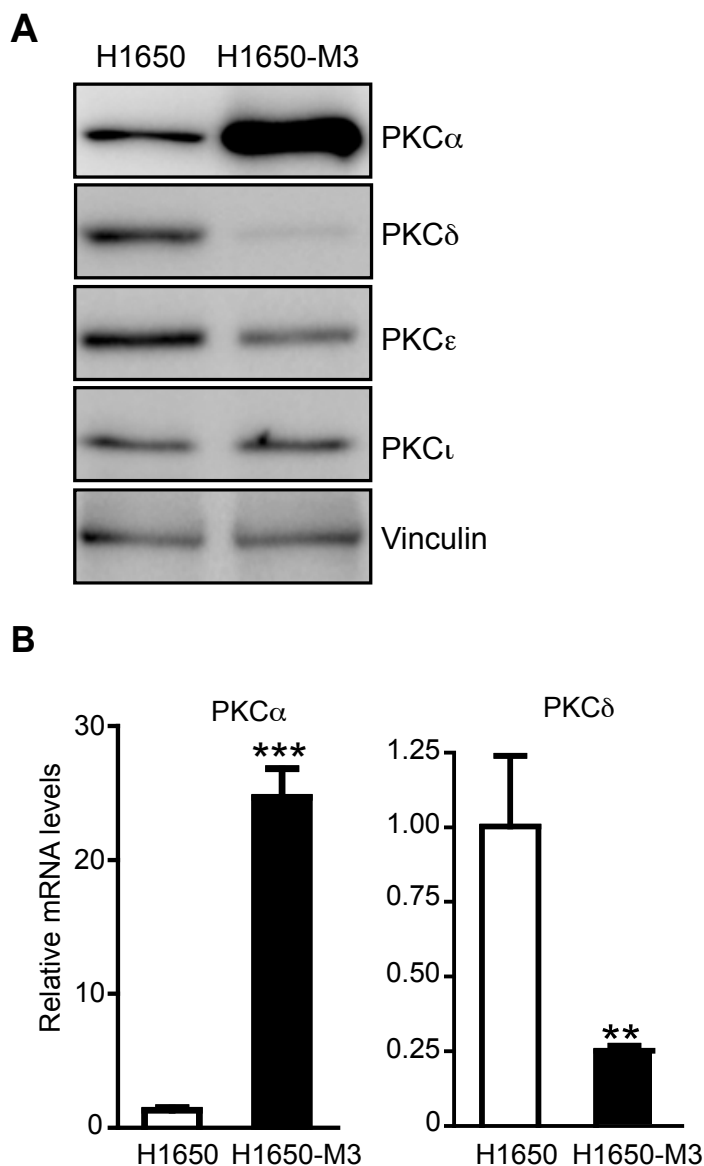


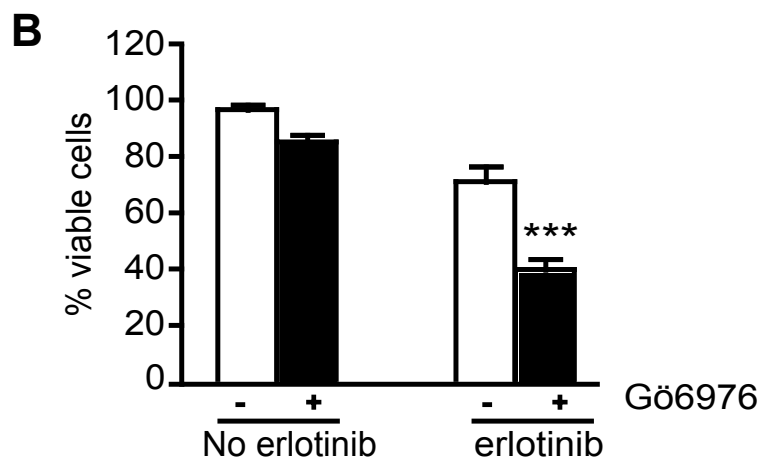
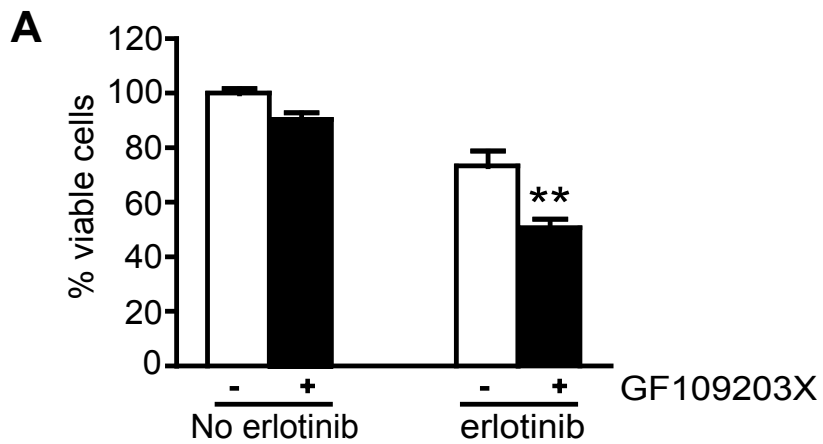
Figure 2.1. Erlotinib-resistant cells are characterized by altered expressions of PKC isozymes

(A) Expression of PKC Isozymes was analyzed in parental (H1650) and erlotinib-resistant (H1650-M3) cells. Protein levels of PKCs were determined by Western blot. (B) mRNA levels of PKC α and PKC δ were measured using real-time quantitative PCR in H1650 and H1650-M3 cells. Human 18S rRNA was used as an endogenous control. Fold-changes in mRNA levels were determined by normalizing to mRNA levels in H1650 using the Δ Ct method. **, $p < 0.01$; ***, $p < 0.001$

In initial experiments (Fig. 2.2A), we observed that treatment of H1650-M3 cells with the pan PKC inhibitor GF 1090203X (5 μ M, added 1 h before and during erlotinib treatment) increases the sensitivity of H1650-M3 cells to erlotinib (10 μ M). Next, we examined the action of Gö6976, which preferentially inhibits cPKCs. As shown in Fig. 2.2B, Gö6976 (5 μ M) also enhances the killing effect of erlotinib in H1650-M3 cells. As PKC α is the only cPKC expressed in these NSCLC cells, these results suggest the possibility that PKC α mediates erlotinib resistance.

To unambiguously establish a role for PKC α in erlotinib resistance, we used RNAi. Two different PKC α RNAi duplexes, were transfected into H1650-M3 cells, which deplete PKC α by > 90 % upon delivery into H1650-M3 cells relative to a non-target control RNAi duplex (Fig. 2.2C, *inset*). A dose-response analysis for inhibition of cell viability by erlotinib treatment established an $IC_{50} = 5.1 \pm 0.8 \mu\text{M}$ (n=3) in control H1650 cells (which is similar to parental H1650 cells, [212] and data not shown). On the other hand, IC_{50} in H1650-M3 cells was > 20 μ M (n=3), as determined in a previous study [212]. Notably, PKC α depletion sensitizes H1650-M3 cells to erlotinib, as determined by the reduction in IC_{50} ($8.7 \pm 1.4 \mu\text{M}$, for PKC α #1 RNAi; $9.2 \pm 3.0 \mu\text{M}$, for PKC α #2 RNAi) (Fig. 2C). To determine if PKC α up-regulation plays a role in inducing erlotinib resistance, we overexpressed PKC α in parental H1650 cells using an adenovirus (Adv). A LacZ Adv was used as control (Fig. 2.2D, *inset*). We found that PKC α overexpression failed to alter the response of H1650 cells to erlotinib ($IC_{50} = 4.7 \pm 1.3 \mu\text{M}$, for PKC α Adv; $IC_{50} = 5.5 \pm 2.0 \mu\text{M}$, for LacZ Adv). Thus, our data indicate that

although PKC α is required for the resistance of NSCLC cells to erlotinib, overexpression of this kinase is not alone sufficient to induce erlotinib resistance.



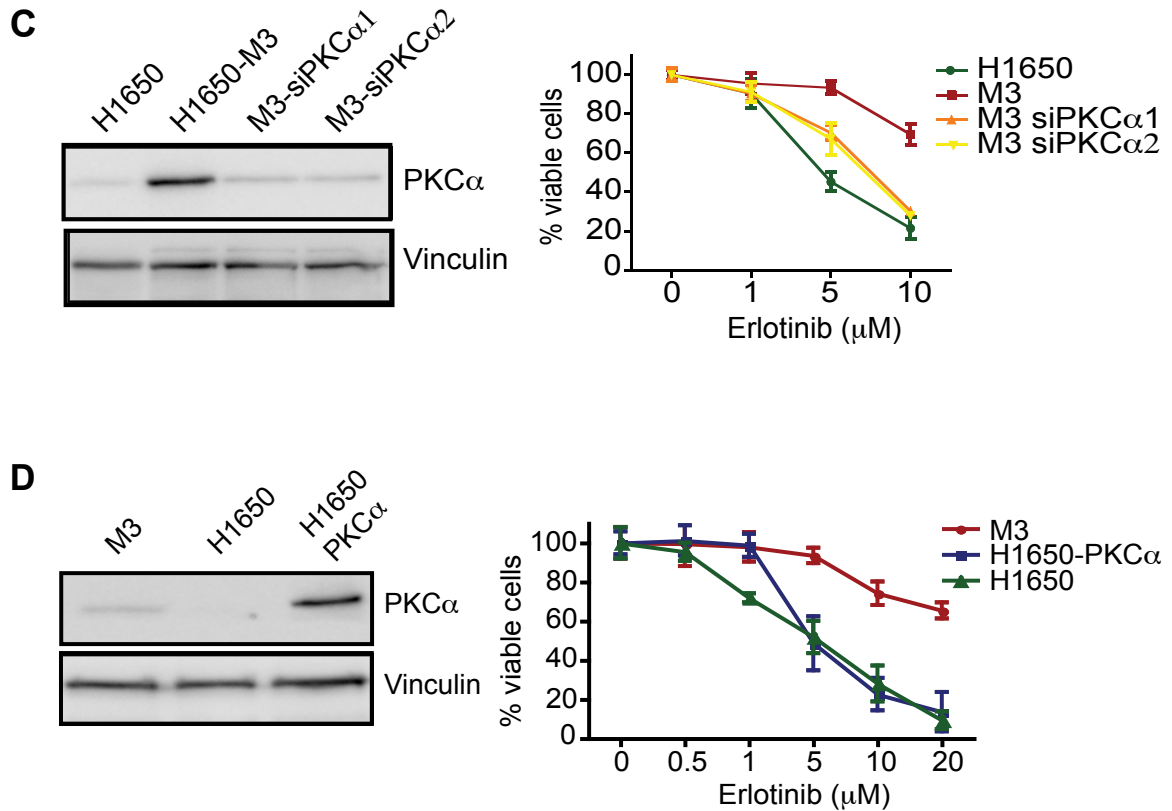


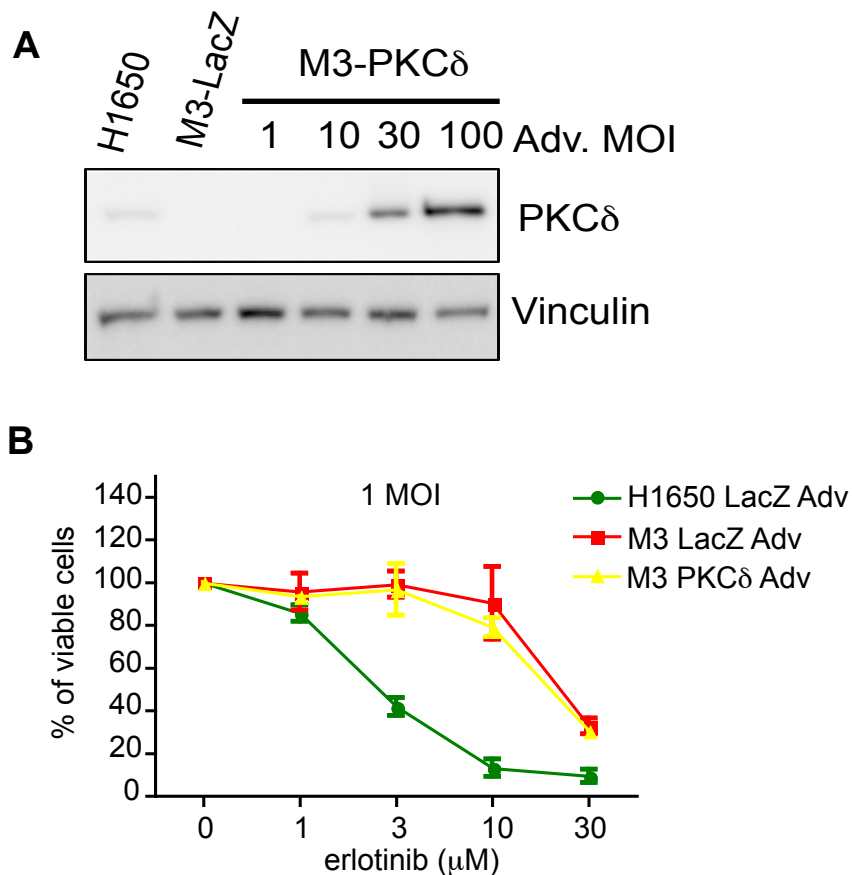
Figure 2.2 PKC α protects H1650-M3 cells from erlotinib induced cell death

(A) Erlotinib-resistant (H1650-M3) cells were pre-treated for 1 h with vehicle or the pan-PKC inhibitor GF109203X (5 μ M). Cells were then treated with erlotinib (10 μ M), and cell viability was determined 24 h later using MTS. **, $p < 0.01$. (B) Erlotinib-resistant (H1650-M3) cells were pre-treated for 1 h with vehicle or a classical PKC inhibitor Gö6976 (5 μ M). Cells were then treated with erlotinib (10 μ M), and cell viability was determined 24 h later using MTS. *** $p < 0.001$. (C) Parental H1650 and erlotinib-resistant (H1650-M3) cells were seeded in 96-well plates. H1650-M3 cells were transfected with either PKC α RNAi duplexes ($\alpha 1$ or $\alpha 2$) or non-target control (NTC). After 48 h, cells were treated with erlotinib at the indicated concentrations. Cell viability was determined 24 h later using MTS. (D) H1650 cells were infected with either LacZ Adv or PKC α Adv (30 MOI). 5 days following infection, H1650-PKC α were seeded in 96-well plates and treated with erlotinib at the indicated concentrations the following day. Cell viability was determined 24 h later using MTS.

PKC δ alters the sensitivity of H1650-M3 cells to erlotinib

Our results clearly ascribe a role for PKC α in determining the sensitivity of H1650 cells to erlotinib. The fact that H1650-M3 cells display PKC δ down-regulation relative to parental cells prompted us to investigate whether changes in PKC δ levels could also dictate the sensitivity to the TKI. It has been reported that PKC δ mediates cytotoxic effect of several anti-cancer drugs [81, 221]. To address this issue, we overexpressed PKC δ in H1650-M3 cells using a PKC δ Adv. A LacZ Adv was used as a control (Fig. 2.3A). As shown in Fig. 2.3B, overexpression of PKC δ in erlotinib-resistant cells caused a reduction in the IC₅₀ for erlotinib. The effect was proportional to the expression levels of PKC δ obtained by infecting cells with different MOIs of the PKC δ Adv. Indeed, infection with an MOI=1 pfu/cell did not cause any significant PKC δ overexpression and consequently failed to sensitize H1650-M3 cells to erlotinib (IC₅₀ = 24.2 \pm 0.6 μ M for PKC δ Adv; IC₅₀ = 24.7 \pm 2.0 μ M for LacZ Adv). On the other hand, an MOI=10 pfu/cell caused a noticeable shift to the left in viability dose-response for the erlotinib (IC₅₀ = 8.7 \pm 1.9 μ M for PKC δ Adv; IC₅₀ = 26.4 \pm 0.4 μ M for LacZ Adv). At higher MOIs, the sensitivity of H1650-M3 cells was essentially similar to that observed in control (LacZ-infected) H1650 cells (MOI=30: IC₅₀ = 6.3 \pm 0.5 μ M for PKC δ Adv; IC₅₀ = 22.2 \pm 0.4 μ M for LacZ Adv; MOI=100: IC₅₀ = 4.5 \pm 0.4 μ M for PKC δ Adv; IC₅₀ = 19.5 \pm 1.0 μ M for LacZ Adv). These results indicate that PKC δ down-regulation contributes to erlotinib resistance of NSCLC cells.

Previous studies have shown that overexpression of one PKC isozyme could alter the expression of PKC family member. For example, overexpression of PKC α could alter the expression of PKC δ , PKC ϵ and PKC ι in different cellular models [107, 154, 198]. As erlotinib-resistant H1650 cells display PKC α overexpression and PKC δ down-regulation relative to the parental cell line, we asked if there was a mutual regulation controlling these changes. To test our hypothesis, we either overexpressed PKC α or depleted PKC δ in the parental H1650 cells.



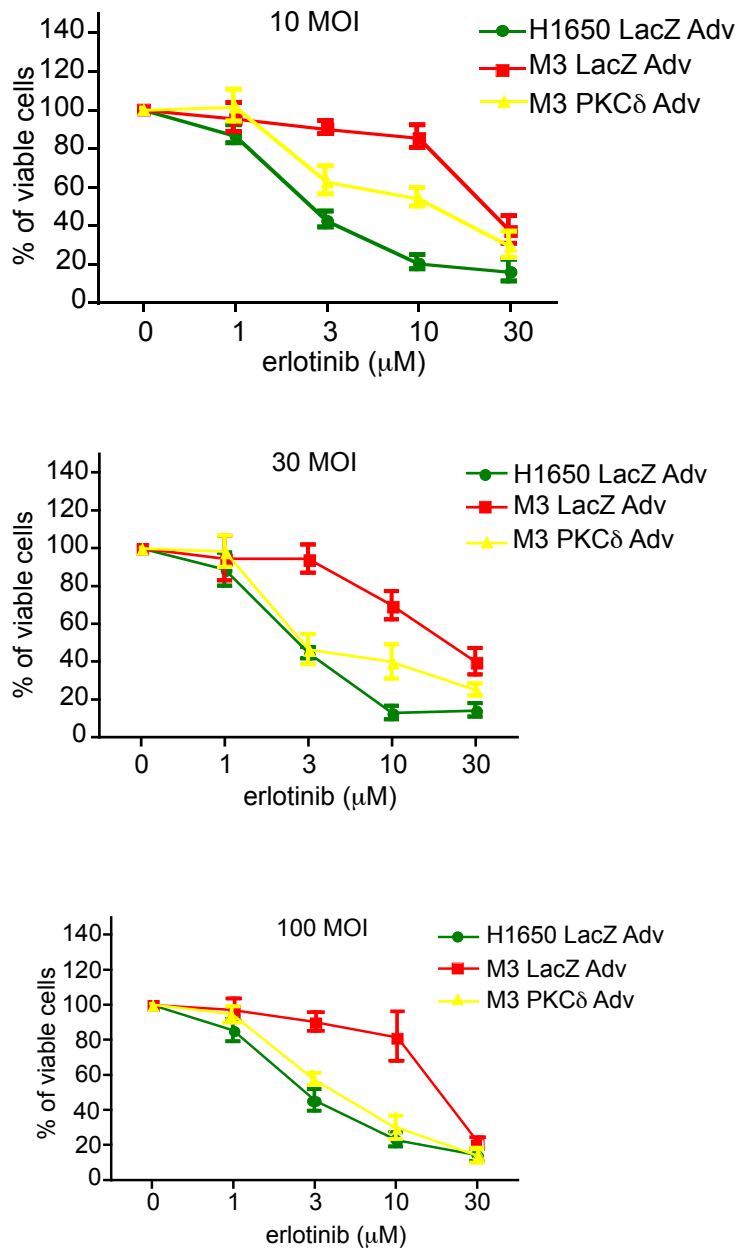
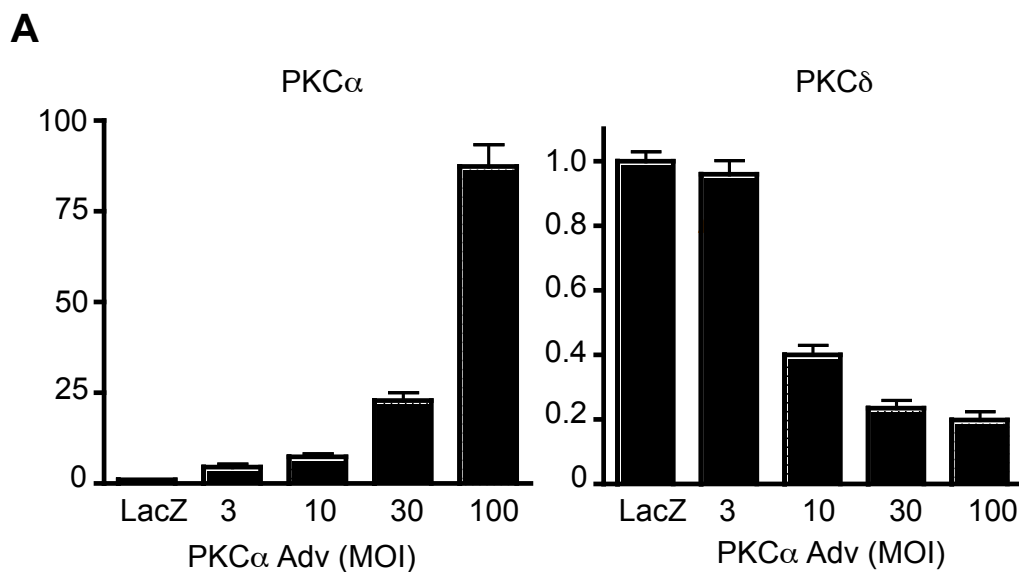


Figure 2.3 PKCδ alters the sensitivity of H1650-M3 cells to erlotinib

(A) Overexpression of PKCδ after 5 days was confirmed using Western blot. (B) H1650-M3 cells were infected with either LacZ Adv or PKCδ Adv at different MOI. 48 h after infection, H1650-M3-PKCδ cells were seeded in 96-well plates, and treated with erlotinib at indicated concentrations 24 h later. Cell viability was determined the following day using MTS.

Interestingly PKC α overexpression by adenoviral means reduced the expression of PKC δ mRNA and protein levels. These effects were proportional to the levels of PKC α overexpression achieved by using increased MOIs of the PKC α Adv (Fig. 2.4A and 2.4B). In the next set of experiments we assessed if down-regulation of PKC δ alters PKC α expression levels. PKC δ expression was silenced from parental H1650 cells using RNAi and PKC α levels were determined by Western blot. As shown in Fig. 2.4C, both control and PKC δ silenced H1650 cells display similar PKC α levels. Furthermore, we overexpressed PKC δ in erlotinib-resistant M3-H1650 cells, and expression levels of PKC α essentially remain unchanged (Fig. 2.4D). These results argue for a unidirectional cross-talk between PKCs whereby overexpression of PKC α in NSCLC cells contributed to the down-regulation of PKC δ , but PKC δ levels were unable to influence those of PKC α .



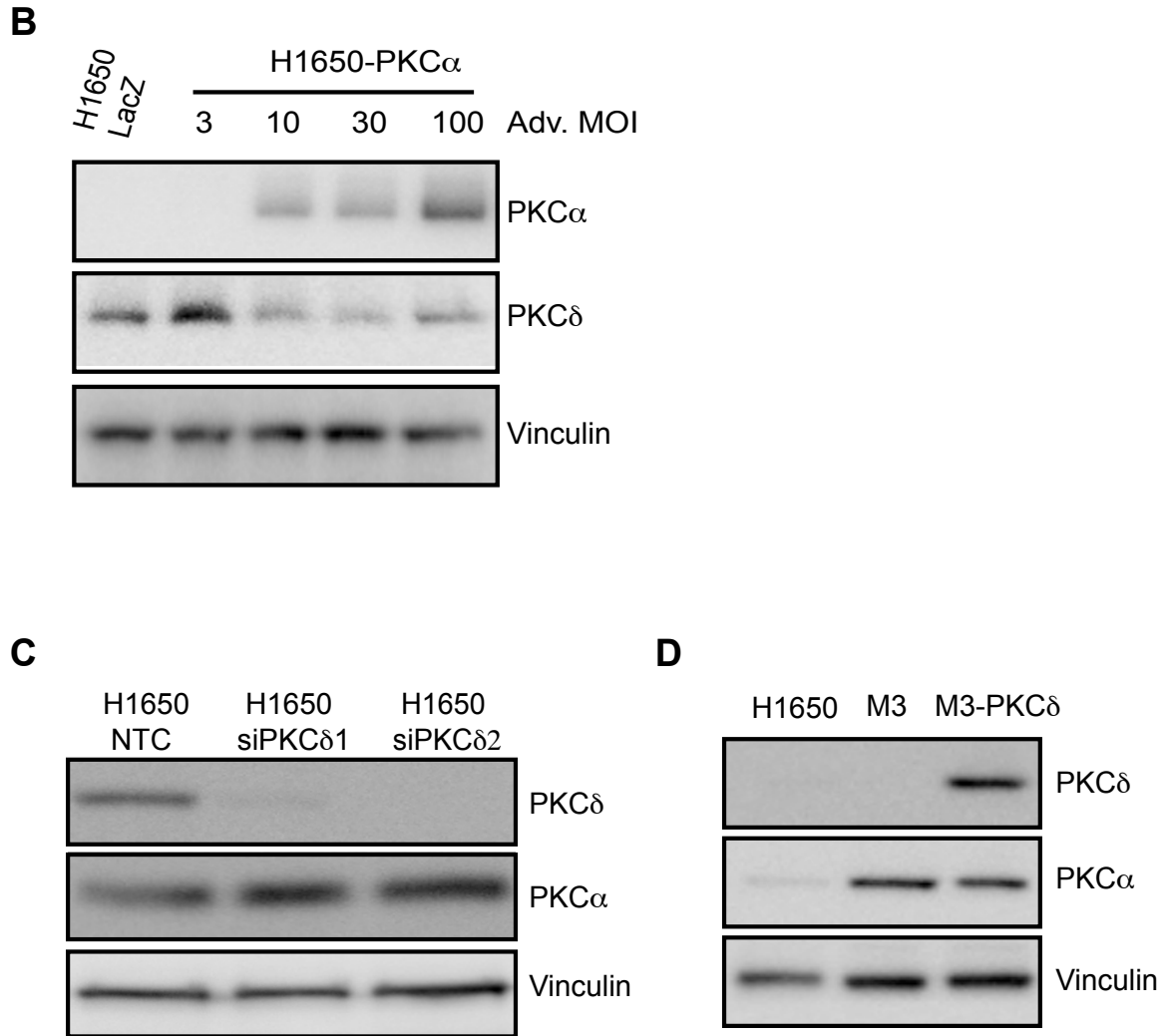


Figure 2.4 PKC α modulates the expression of PKC δ

(A) H1650 cells were infected with either LacZ Adv (100 MOI) or PKC α Adv at different MOI. Changes in PKC α , and PKC δ mRNA were determined by qPCR 72 h after infection. Results are expressed as fold-change relative to LacZ Adv. (B) Protein levels of PKC α and PKC δ were analyzed by Western blot 72 h after infection. (C) Parental H1650 cells were transfected with either PKC δ RNAi duplexes (δ 1 or δ 2) or non-target control (NTC) RNAi duplexes. Expression of PKC α and PKC δ were analyzed by Western blot after 72 h. (D) H1650-M3 cells were infected with either LacZ Adv or PKC δ Adv (100 MOI). The expressions of PKC δ and PKC α were measured in H1650, H1650-M3 and PKC δ overexpressing H1650-M3 cells after 96 h using Western blot.

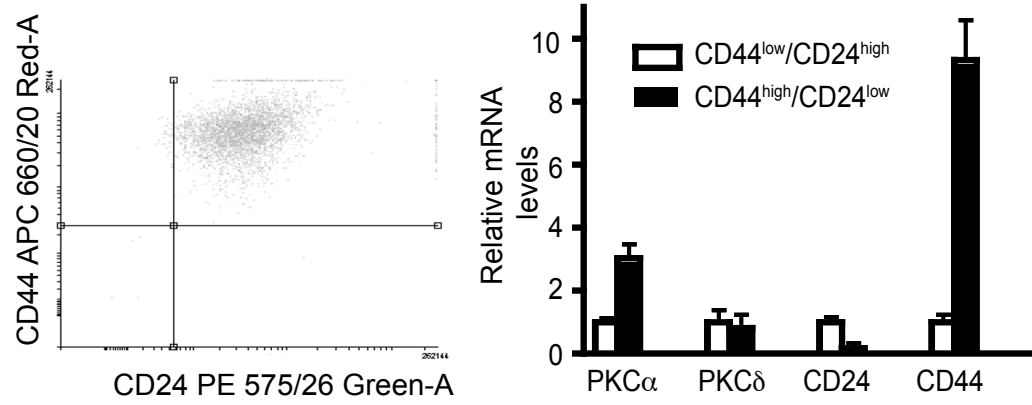
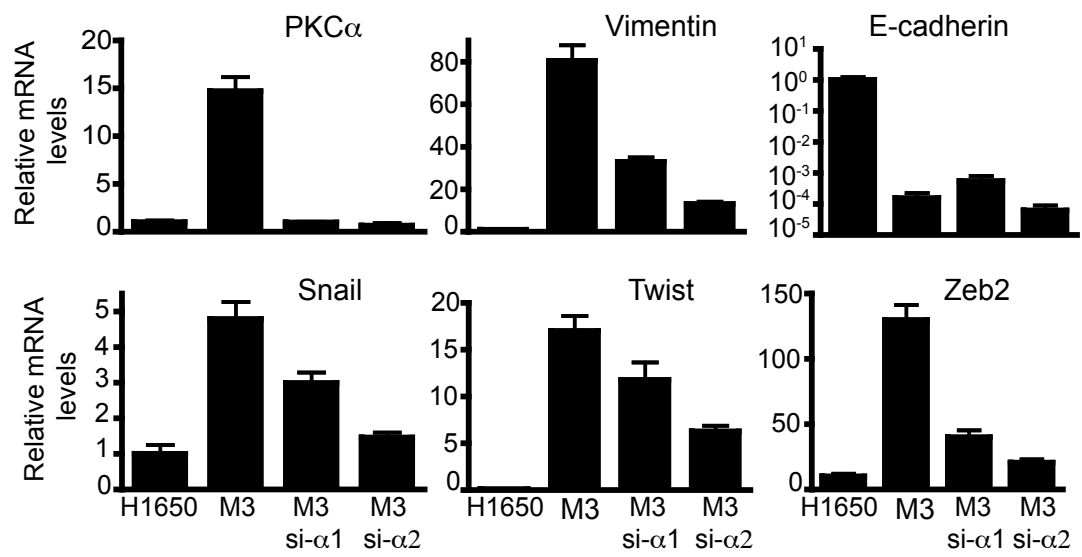
PKC α is required for the maintenance of H1650-M3 cell mesenchymal phenotype

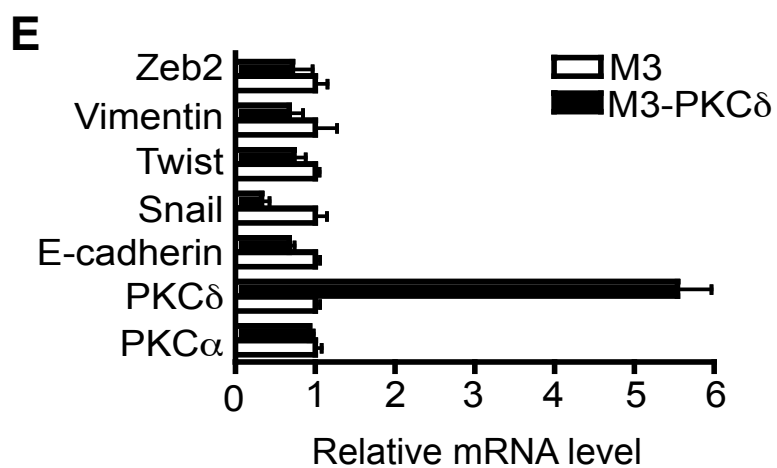
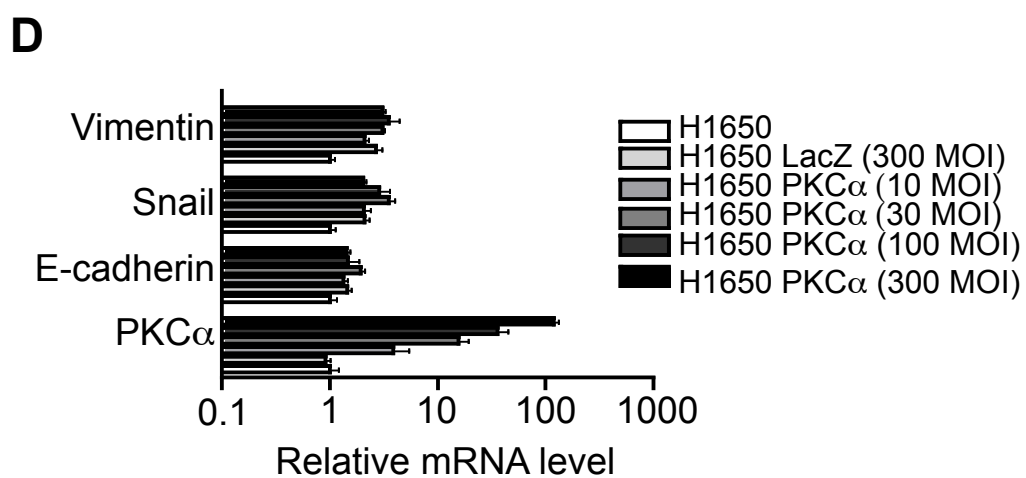
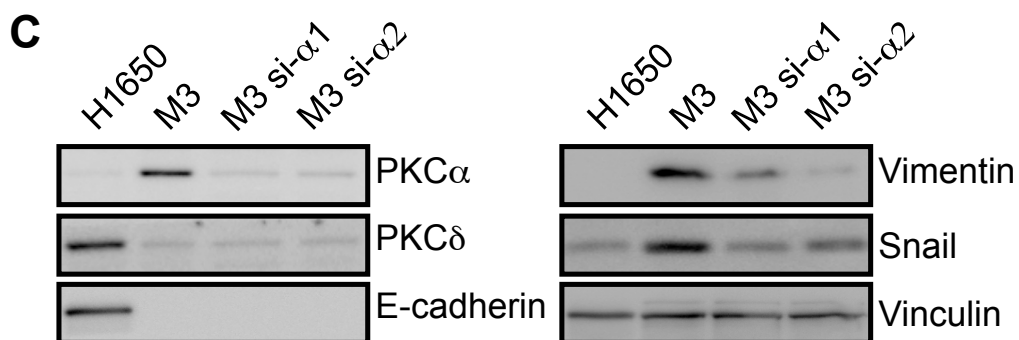
Erlotinib-resistant H1650 cells exhibit mesenchymal properties, a phenotype that is mediated by TGF- β [212]. The mesenchymal phenotype is a hallmark of cancer stem cells exhibiting aggressive phenotype [182]. A recent study in breast cancer showed that PKC α is up-regulated in cells that had undergone EMT [181], thus we speculated that this kinase might contribute to the maintenance of the mesenchymal phenotype of erlotinib-resistant cells. Parental H1650 cells were sorted into CD44^{high}/CD24^{low} and CD44^{low}/CD24^{high} enriched populations, and PKC α mRNA levels were determined in both populations using qPCR. This experiment shows that PKC α mRNA levels were elevated in the CD44^{high}/CD24^{low} cells (Fig. 2.5A).

As shown in a previous study [212], H1650-M3 cells display markedly elevated levels of genes associated with EMT, including vimentin, Snail, Twist, and Zeb as well as reduced levels of E-cadherin. To establish a potential involvement of PKC α up-regulation in the mesenchymal phenotype of H1650-M3 cells, we examined the expression of EMT markers after silencing PKC α . Notably, PKC α RNAi depletion markedly reduced vimentin, Snail, Twist, and Zeb levels, suggesting that this kinase mediates the induction of these EMT genes. Levels of the epithelial marker E-cadherin remained unaffected (Fig. 2.5B). For those markers that could be readily detected by Western blot (vimentin and Snail), changes were also validated at the protein level (Fig. 2.5C). Despite the PKC α requirement for the expression of EMT markers, it became apparent that overexpression of this kinase in the parental H1650 cells was not sufficient

to induce these EMT genes, as determined by qPCR 72 h after infection with increased MOIs of the PKC α Adv (Fig. 2.5D). No changes were observed even 1 week after PKC α Adv infection. Altogether, these results indicate that PKC α is required for the maintenance of mesenchymal phenotype of erlotinib-resistant cells, however its overexpression is insufficient to induce this phenotypic change.

Next, we set to explore whether PKC δ has a role in the expression of genes associated with EMT transition. As PKC δ is down-regulated in H1650-M3 cells, we adenovirally overexpressed PKC δ in these cells and assessed the expression of EMT markers by qPCR. Unlike PKC α silencing, ectopic overexpression of PKC δ in H1650-M3 cells did not change the expression of vimentin, Twist, or Zeb2, although a reduction in Snail levels could be observed. Likewise, PKC δ overexpression did not affect E-cadherin mRNA levels (Fig. 2.5E). Consistent with these results, no changes in protein levels for E-cadherin and vimentin could be observed (Fig. 2.5F). We also found that PKC δ RNAi depletion from parental H1650 cells failed to change the expression of EMT markers (Fig. 2.5G). Therefore, the involvement of PKC δ is only confined to erlotinib resistance but not to EMT.

A**B**



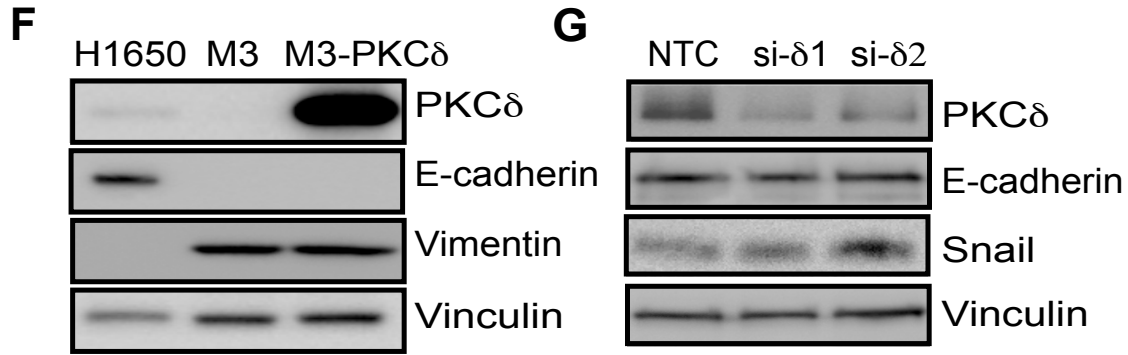


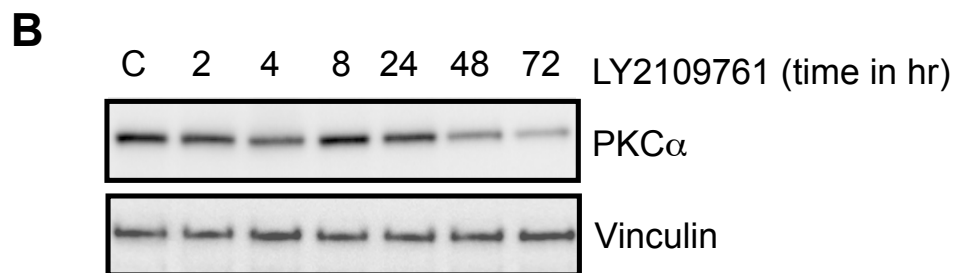
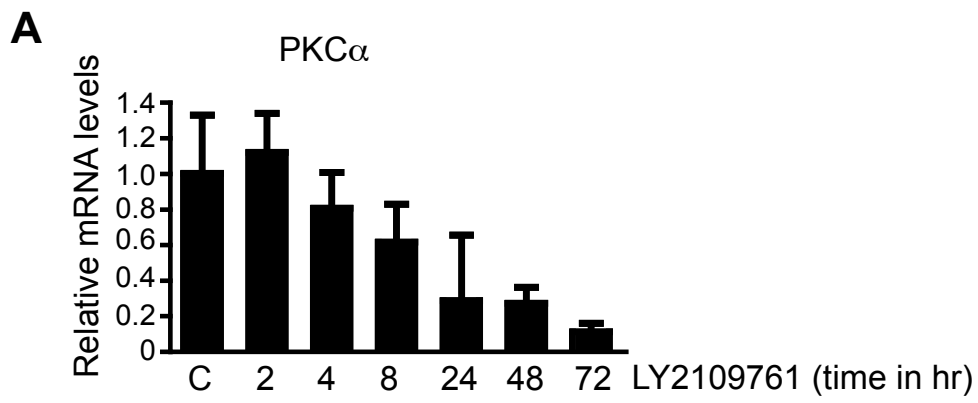
Figure 2.5 PKC α is required but not sufficient for maintenance of mesenchymal phenotype

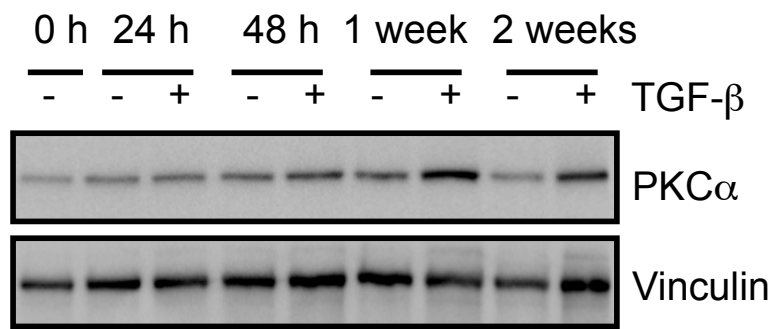
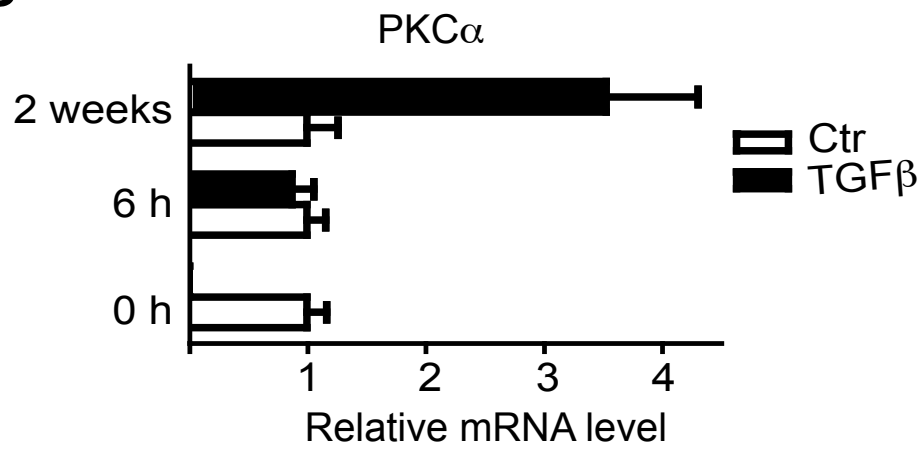
(A) Parental H1650 cells were analyzed by flow cytometry for CD44 and CD24 expressions. Following the analysis, cells were sorted into CD44^{high}/CD24^{low} and CD44^{low}/CD24^{high} sub-populations and the levels of PKC α and PKC δ mRNA were compared by qPCR in each population. (B) H1650-M3 cells were transfected with either PKC α RNAi (α 1 or α 2) or non-target control (NTC) RNAi duplexes. After 72 h, RNA was extracted for qPCR analysis of selected genes associated with epithelial or mesenchymal phenotypes. Results are expressed as fold-change relative to parental H1650 cells. (C) Proteins were extracted after PKC α RNAi depletion and levels of epithelial and mesenchymal markers were determined using Western blot. (D) H1650 cells were infected with either LacZ Adv (100 MOI) or PKC α Adv at different MOI. After a week, expression mesenchymal genes and E-cadherin were determined using qPCR. (E) H1650-M3 cells were infected with either LacZ Adv or PKC δ Adv (100 MOI). After 96 h, the mRNA level of mesenchymal associated genes was measured. (F) Western blot showing the protein expression of PKC δ , Vimentin and E-cadherin. (G) Parental H1650 cells were transfected with either PKC δ RNAi (δ 1 or δ 2) or non-target control (NTC) RNAi duplexes. Expression of PKC δ , E-cadherin and Snail were analyzed by Western blot after 72 h.

PKC α up-regulation in erlotinib-resistant cells is mediated by TGF- β

TGF- β has been widely implicated in EMT in multiple cancer types [35, 88, 201]. Activation of the TGF- β signaling pathway mediates EMT and erlotinib resistance in H1650 cells [212]. Based on this premise, we sought to establish if a causal relationship exists between TGF- β signaling and PKC α expression. H1650-M3 cells were treated with the TGF- β inhibitor LY2109761 for different times, and its efficacy to inhibit TGF- β signaling was confirmed by its ability to reduce Smad2 phosphorylation (data not shown). Notably, the TGF- β inhibitor caused a time dependent reduction in PKC α mRNA levels (Fig. 2.6A). This effect was noticeable at the protein level particularly 48 and 72 h after LY2109761 treatment (Fig. 2.6B). Furthermore, we treated parental H1650 cells with recombinant TGF- β for different times, and found that it caused a significant PKC α up-regulation both at mRNA and protein levels. This effect was quite remarkable after long term treatment with TGF- β (Fig. 2.6C and 2.6D). Therefore, TGF- β signaling is implicated in the overexpression of PKC α in erlotinib-resistant cells.

Lastly, we sought to establish an association between PKC α up-regulation and TGF- β signaling in the induction of the mesenchymal phenotype. H1650 cells were infected with the PKC α Adv (or LacZ Adv as a control) and then subject to TGF- β treatment. mRNA was extracted a week after treatment and EMT markers were determined by qPCR. As shown in Fig. 2.6E, overexpression of PKC α potentiated the induction of the mesenchymal genes vimentin, Snail and Twist, by TGF- β .



C**D**

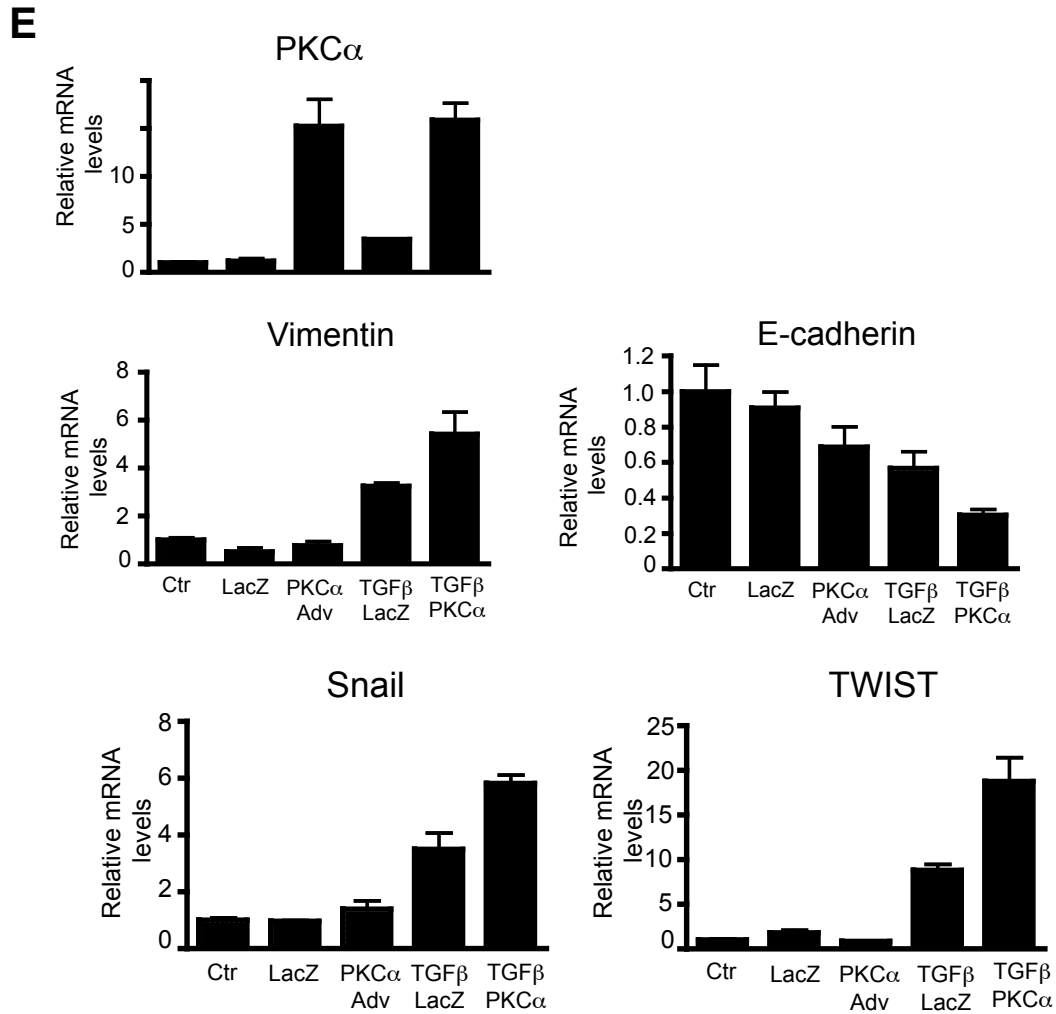


Figure 2.6 TGF- β signaling controls expression of PKC α in erlotinib-resistant cells

(A) Erlotinib-resistant (H1650-M3) cells were plated overnight and treated with a TGF- β inhibitor (LY2109761, 5 μ M) for the indicated times. PKC α mRNA levels were measured by qPCR. (B) Protein levels of PKC α were determined by Western blot and densitometric analysis was carried out as described before. (C) Parental H1650 cells were cultured on a 60 mm plate and treated with TGF- β (5 η g/ml) for 24 h, 48 h, 1 week or 2 weeks. PKC α protein levels were determined using Western blot. (D) mRNA levels of PKC α were determined after 6 h and 2 weeks of TGF- β treatment. (E) H1650 cells were infected with either LacZ Adv or PKC α Adv (30 MOI). 24 h after infection, H1650-PKC α cells were treated with TGF- β (5 η g/ml) for a week. mRNA expressions for PKC α and genes associated with epithelial and mesenchymal phenotypes were assessed using qPCR.

2.3 Discussion

Tumors harboring activating mutations of EGFR depend on the oncogene to maintain their malignant proliferation and survival. TKIs such as erlotinib have been effective for treatment of NSCLC patients with EGFR activating mutations. However, many of the patients exposed to erlotinib develop acquired drug resistance to the targeted-molecular therapy. One of the main causes of drug resistance is a relief of EGFR addiction and dependence on other oncogenic alterations. Characterization of signaling pathways implicated in the new oncogenic alteration is important to determine an effective therapy for patients with acquired resistance. PKC isozymes have been recognized as key effectors of known oncogenes such as *c-MET*, *KRAS* and *TGF- β* , which are implicated in induction of drug resistance [14, 21, 132]. Moreover, phorbol esters, which are known activators of PKCs, have been shown to induce multidrug resistance in cellular and xenografts models. In this regard, early studies in breast and small cell lung cancer have identified a role of phorbol esters in inducing phosphorylation of drug efflux pumps leading to reduced drug accumulation [48].

Here we present evidence for the role of PKC isozymes in the mechanisms of erlotinib resistance and epithelial-mesenchymal transition in NSCLC cells. Using isogenic cell models, we found correlations between alteration in PKC isozymes expressions and induction of resistance to erlotinib. Indeed, erlotinib-resistant H1650-M3 cells exhibit high levels of PKC α while PKC δ expression was down-regulated. Although this is the first evidence for the involvement of the two PKC isozymes in resistance to molecular-targeted therapy, altered expressions of PKC α and PKC δ have been detected

in several cancer cells resistant to endocrine therapy and chemotherapeutic drugs. Elevation of PKC α expression or activity has been reported in pancreatic, colon, glioma and gastric cancer cells resistant to chemotherapeutic drugs such as cisplatin, doxorubicin and vincristine [30, 114, 207, 220]. Interestingly, comparable to what is observed in erlotinib-resistant cells, continuous exposure of MCF-7 cells to Tamoxifen rendered high levels of PKC α and down-regulation of PKC δ [102].

Studies have indicated the importance of PKC α overexpression in protecting cancer cells against drug-induced cell death. PKC α overexpression in colon cancer cells attenuated doxorubicin induced apoptosis by elevating phosphorylation of Bcl-2, Bad and decreasing PARP cleavage. PKC α has also been shown to regulate the cell cycle kinase cyclin D in drug resistant pancreatic cancer cells. More importantly, in several drug resistant cancer models, PKC α overexpression has been associated with reduced drug resistance by inducing increased expression and phosphorylation of a drug efflux pump, P-gp, encoded by the multidrug resistant gene 1 (*MDR1*) [99]. The functional importance of PKC α overexpression has further been demonstrated by using pharmacological inhibitors and RNA interference. Inhibition of PKC α using Gö6976 restored the sensitivity of pancreatic cancer cells to chemotherapeutic drugs [30]. Blocking PKC α by RNAi interference also reversed drug resistance in ovarian cancer cells [220]. In our study, we found that RNAi depletion or inhibition of PKC α using Gö6976 sensitize erlotinib-resistant NSCLC cells to the TKI.

Overexpression of one PKC isozyme could lead to altered expression of other members of the PKC family and there are several potential reasons suggested for the

observed phenomenon. Overexpression of one PKC isozyme could compete for proteases, which are required for degradation of other PKCs. Alternatively cross-phosphorylation between PKCs, could change the sensitivity to proteolysis and result in mRNA stability [154]. In other cases, the alteration in more than one PKC isozymes occurs as cross-regulation by several PKCs is required to achieve a full transformed phenotype. For example, as shown in MCF-7 cells, overexpression of PKC α resulted in down-regulation of endogenous PKC δ and reduction in PKC δ expression as well as high levels of PKC α were found to be important for the phenotypic changes observed in MCF-7-PKC α cells [198]. Similar phenomenon was observed in the H1650 where overexpression of PKC α resulted in down-regulation of PKC δ comparable to the levels in erlotinib-resistant H1650-M3 cells. Moreover, we found that the overexpression of PKC α as well as down-regulation of PKC δ were important to maintain erlotinib resistance in H1650-M3 cells.

As previously characterized, H1650-M3 cells have elevated expression of genes associated with EMT and display morphological changes that are reminiscent of mesenchymal phenotype. Interestingly, the parental erlotinib naive cells were shown to possess a subpopulation of cells that are mesenchymal, erlotinib-resistant and similar to H1650-M3 cells [212], indicating that H1560-M3 cells were potentially generated through a selection process that favors the survival of cells that utilize alternate mechanisms to overcome drug-induced death. Certainly the identification of these mechanisms is crucial to effectively target drug-resistant tumors. PKC α has been implicated in the survival of cancer stem cells, which are known to arise during EMT

[181]. Inhibition of PKC α was shown to preferentially induce apoptosis of breast cancer stem cells. Additionally, elevated PKC α expression was found in a subpopulation of normal mammary epithelial cells enriched with a mesenchymal surface marker CD44 [181]. Similarly, our results indicate a correlation between enrichment of mesenchymal phenotype and PKC α expression. Inhibition of PKC α in H1650-M3 cells also led to inhibition of genes associated with mesenchymal phenotype. Interestingly, although exposure to erlotinib resulted in differential expression of EMT markers including up-regulation of mesenchymal genes such as vimentin, snail, twist and Zeb2, and downregulation of the epithelial marker E-cadherin, the effect of inhibiting PKC α was limited to the genes associated with mesenchymal phenotype underscoring its role in the maintenance of this phenotype.

Several factors could regulate the expression of PKC α in NSCLC cells. As TGF- β signaling was shown to be sufficient and required for the induction of erlotinib resistance and EMT in H1650-M3 cells [212], we explored a potential relationship between TGF- β and PKC α . Inhibition of TGF- β signaling blocked the expression of PKC α in H1650-M3 cells. Moreover, treatment with TGF- β increased the expression of PKC α in the parental erlotinib sensitive epithelial cells indicating that in the process of acquiring an aggressive phenotype, TGF- β up-regulated the expression of PKC α . Although the promoter region of PKC α does not consist of a TGF- β binding region, studies have identified PKC α as a TGF- β target gene. For example, gene profiling in A549 lung adenocarcinoma cell lines has shown that *Prkca* is one of the genes up-regulated by TGF- β treatment [142]. In conclusion our results provide evidence for a role

of PKCs in acquired resistance and EMT and suggest a potential use of PKC α inhibitors to specifically target mesenchymal tumors including cancer stem cells that are resistant to therapy.

2.4 Materials and Methods

Reagents

Erlotinib hydrochloride was purchased from Santa Cruz (Santa Cruz, CA). The pan-PKC inhibitor GF109203X was purchased from Enzo Life Sciences (Plymouth Meeting, PA). The cPKC inhibitor Gö6976 was obtained from LC laboratories (Woburn, MA). The CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit was purchased from Promega.

Cell Culture

H1650 and H1650-M3 cell lines were generated in the laboratory of Dr. Raffaella Sordella (Cold Springs Harbor, NY) [212] and cultured in RPMI 1640 medium supplemented with 5 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37 °C in a humidified 5 % CO₂ atmosphere.

Real-time PCR

Total RNA was extracted from subconfluent cell cultures using RNeasy Kit from Qiagen (Valencia, CA). Total RNA (1 µg) was reverse transcribed to cDNA using Taqman reverse transcription reagent kit in a total volume of 20 µl (Applied Biosystems, Branchburg, NJ). Real-time quantitative PCR (qPCR) was performed using an ABI PRISM 7700 detection system. The reaction was carried out in triplicate containing TaqMan universal PCR MasterMix (Applied Biosystems), target primers (300 nM), fluorescent probe (200 nM), and 4 µl of transcribed cDNA (6X dilution). Taqman

primers 5'end-labeled with 6-carboxyfluorescein (FAM) for PKC α , PKC δ , E-cadherin, Snail, Twist1, Vimentin, Zeb2 and 18S rRNA (housekeeping gene) were used. PCR product amplification was continuously monitored using the sequence detection system software version 1.7 (Applied Biosystems). Triplicate cycle threshold (Ct) values were averaged and normalized to an average 18S Ct value to calculate the Δ Ct. The Δ (Δ Ct) was determined by subtracting the control Δ Ct value from the experimental Δ Ct value. Fold change were expressed as $2^{-\Delta\Delta Ct}$.

Western blot

Cells were harvested in lysis buffer (125 mM Tris-HCl, pH 6.8, 50% glycerol, 4% SDS, 0.08% bromophenol blue, and 20 % β -mercaptoethanol). Samples were resolved in 10 % SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MD). After blocking with 5 % milk in 1 % Tween 20/PBS, membranes were incubated with the primary antibody. The following primary antibodies were used: anti-PKC α (EMD Millipore Corp., Billerica, MA), anti-PKC ϵ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PKC ι (Abcam, Cambridge, MA), anti-PKC δ , anti-vimentin, anti-E-cadherin, anti-Snail, anti-phospho-Smad2 (Cell Signaling Technology, Danvers, MA), and anti-vinculin (Sigma-Aldrich, St. Louis, MO). Either anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA) were used. Bands were visualized by the enhanced chemiluminescence Western blotting detection system, and images were captured using the FujiFILM LAS-3000 system.

RNA interference

RNAi duplexes for silencing PKC α were purchased from Dharmacon (Lafayette, CO). The target sequences were: PKC α RNAi #1; CCAUCCGCUCCACACUAAA; PKC α RNAi # 2; GAACAAGGAAUGACUU [134]. Control silencer RNAi was purchased from Ambion (Austin, TX). Cells were transfected with RNAi duplexes (25 nM) using Lipofectamine RNAi/MAX and used for the indicated experiments.

Adenoviral infections

Cells were infected with adenoviruses (Advs) for PKC α , PKC δ , or LacZ (control) using different multiplicities of infection (MOIs) as previously described [184]. The infection was carried out in RPMI 1640 medium supplemented with 2 % FBS. Complete medium was added 4 h later. Experiments were carried out 72-96 h later, as indicated.

Cell viability assay

Cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit, a colorimetric assay that contains MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and phenazine ethosulfate (PES) with enhanced chemical stability. Cells seeded into 96-well plates (1×10^4 cells/well) were treated with different concentrations of erlotinib for different times, as indicated. One h after addition of the One Solution Reagent, absorbance was recorded at 490 nm using a 96-well plate reader.

Flow Cytometry

Subconfluent H1650 cells were detached using 0.02 % EDTA in PBS and washed, pelleted and resuspended in FACS buffer (PBS pH 7.2, 0.2 % bovine serum albumin). 5×10^6 cells were co-stained with phycoerythrin-conjugated anti-human CD24 and allophycocyanin-conjugated anti-human CD44 (BD Biosciences, San Jose, CA) antibodies. Labeling was performed for 1 h at room temperature in the dark. Labeled cells were washed three times with the FACS buffer and sorted using a BD FACS Aria II cell sorter. Gates were set either at CD44/CD24 high or low expressions, and subpopulation of cells were collected in FACS buffer for RNA extraction.

Statistical Analysis

All statistical analyses were done using GraphPad Prism v5.03 (GraphPad Software, Inc., San Diego, CA). A *p* value < 0.05 was considered statistically significant.

CHAPTER 3: PKC α signaling is required for NF- κ B activation in NSCLC cells

3.1 Introduction

Inflammation is a part of innate immunity in response to physiological and environmental stimuli [109]. However, persistent inflammation causes prolonged activation of immune cells, which results in tissue damage and risks that lead to neoplasia. Induction of proinflammatory cytokines, such as $\text{TNF}\alpha$ or $\text{IL-1}\beta$ activates the transcription factor $\text{NF-}\kappa\text{B}$ through the canonical pathway. The activation process involves various signaling molecules and converges on the $\text{I}\kappa\text{B}$ kinase (IKK) complex, which consists of two closely related kinases, $\text{IKK}\alpha$ and $\text{IKK}\beta$, and the regulatory unit NEMO. Phosphorylation and activation of this complex, particularly $\text{IKK}\beta$, recruits and phosphorylates the $\text{NF-}\kappa\text{B}$ inhibitor protein, $\text{I}\kappa\text{B}\alpha$, at serine residues followed by ubiquitination and subsequent degradation of the inhibitor protein. Loss of $\text{I}\kappa\text{B}\alpha$ releases $\text{NF-}\kappa\text{B}$, which then translocate to the nucleus and drives transcription of multitude of genes. $\text{NF-}\kappa\text{B}$ is an important player in tumor progression as it is activated in chronic inflammation. In addition to the tumor-promoting role of $\text{NF-}\kappa\text{B}$ that involves stimulation of cell cycle progression, inhibition of apoptosis, increased invasive growth, and elevated resistance to radiation and chemotherapy, this transcription factor also participates in tumor initiation. For example, activated $\text{NF-}\kappa\text{B}$ in macrophages and neutrophils stimulates the production of reactive oxygen species (ROS) by inducing the transcription of inducible nitric oxide synthase (iNOS). ROS induce DNA damage and initiates lesions in the surrounding cells, thus contributing to tumorigenesis [74].

IL-1 β is a pro-inflammatory cytokine that triggers a cascade of inflammatory reactions through NF- κ B dependent expression of genes, which also have implications in cancer progression. IL-1 β is produced as an inactive precursor, pro-IL-1 β , in response to injuries or infections. Following induction of pro-IL-1 β expression, the precursor is loaded onto a multi-protein scaffold complex called the inflammasome, where it is cleaved by the pro-inflammatory protease caspase-1 and released out of the cells. The release of IL-1 β into the tumor microenvironment leads to activation of inflammatory signals in the tumor cell and its stroma [167]. Notably, high levels of IL-1 β are found in serum from patients with chronic lung infections and lung cancer [16, 223]. Moreover, exposure of lung epithelial cells to IL-1 β increases expression of cyclooxygenases, metalloproteases and growth factors known to induce proliferation, angiogenesis and invasive phenotype of lung cancer cells [31, 164].

PKCs isozymes are pleiotropic regulators of cellular processes in lung cancer. Nevertheless, their functions are highly dependent on the cell type, occurrence of oncogenic mutations and stimuli. In the present study, we established PKC α signaling as an important component of NF- κ B activation in NSCLC cells. Depletion of PKC α significantly attenuated IL-1 β induced NF- κ B pathway activation, including I κ B phosphorylation, as well as nuclear translocation and DNA binding. Overall, these findings underscore the importance of PKC α signaling in the pathogenesis of lung cancer.

3.2 Results

PKCs regulate NF- κ B activation in NSCLC cells

Tumor infiltration of immune cells due to chronic inflammation marks the progression of many advanced cancer types. NF- κ B is a well-known mediator of responses by proinflammatory cytokines such as TNF α and IL-1 β in NSCLC. As PKC signaling has been linked to NF- κ B activation in several cancer types [54, 124], we set out to determine if PKC signaling contributes to NF- κ B activation in response to inflammatory cytokines in NSCLC. We first measured NF- κ B activation in H460 cells in response to cytokines (TNF α , IL-1 β) known to induce NF- κ B transcriptional activity using a luciferase reporter assay. Stimulation of H460 cells with IL-1 β or TNF α led to an elevation in NF- κ B luciferase activity with the highest luciferase activity being measured 6 h after IL-1 β treatment. Next, to establish the role of PKCs in IL-1 β or TNF α -induced NF- κ B activation, we employed the pan PKC inhibitor GF1090203X to determine the effect of NF- κ B transcriptional activity. Pre-treatment GF1090203X markedly attenuated cytokine-induced transcriptional activation of NF- κ B. Notably, GF1090203X was most effective in inhibiting the activation of NF- κ B in response to IL-1 β than TNF α or PMA (Fig. 3.1).

PKC α is required for IL-1B mediated NF- κ B activation

PKC α and PKC ϵ are found to be overexpressed in H460 cells compared to the normal lung epithelial cells (data not shown). As phosphorylation of I κ B α is a critical

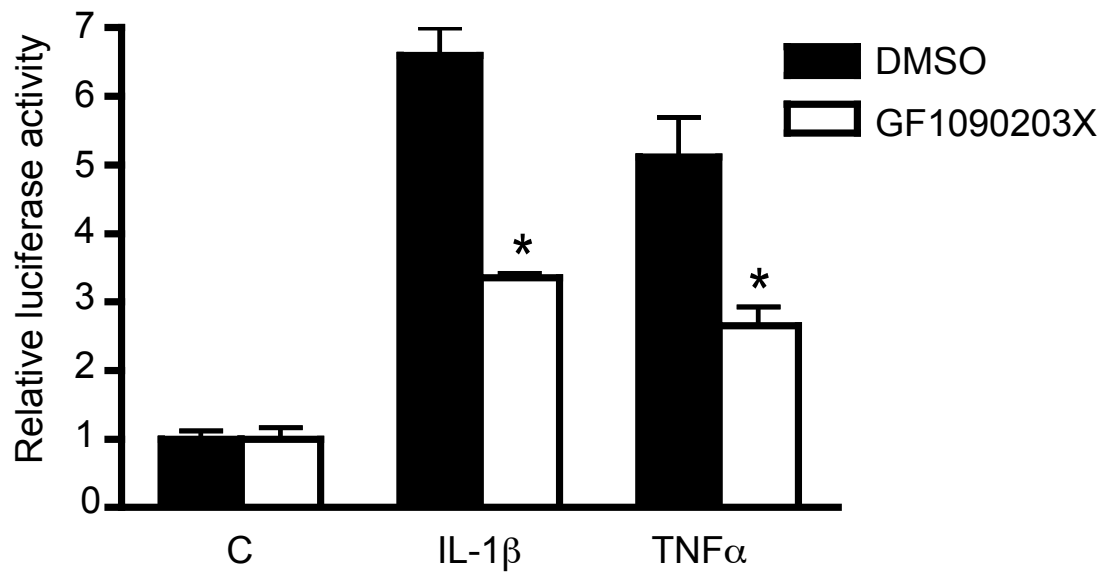


Figure 3.1 PKCs are implicated in NF- κ B activation

H460 cells were co-transfected with NF- κ B firefly luciferase reporter and pTK-*Renilla* plasmid. 24 h later, cells were pre-treated with GF1090203X (5 μ M, 1 h) followed by treatment with TNF α (10 ng/mL, 6 h), IL-1 β (10 ng/mL, 6 h) or vehicle.

step in the activation of the NF- κ B pathway, we next assessed the effect of PKC α or PKC ϵ inhibition on phospho-I κ B α levels. IL-1 β -induced I κ B α phosphorylation was significantly attenuated by pre-treatment with either GF1090203X or a classical PKC inhibitor, Gö6976, thus implicating the involvement of PKC α in IL-1 β induced I κ B α phosphorylation (Fig. 3.2A). To unambiguously demonstrate the involvement of PKC α in regulating I κ B α phosphorylation, we used two different RNAi duplexes to silence PKC α expression from H460 cells. PKC α depletion significantly reduced IL-1 β -induced I κ B phosphorylation and prevented I κ B degradation (Fig. 3.2B).

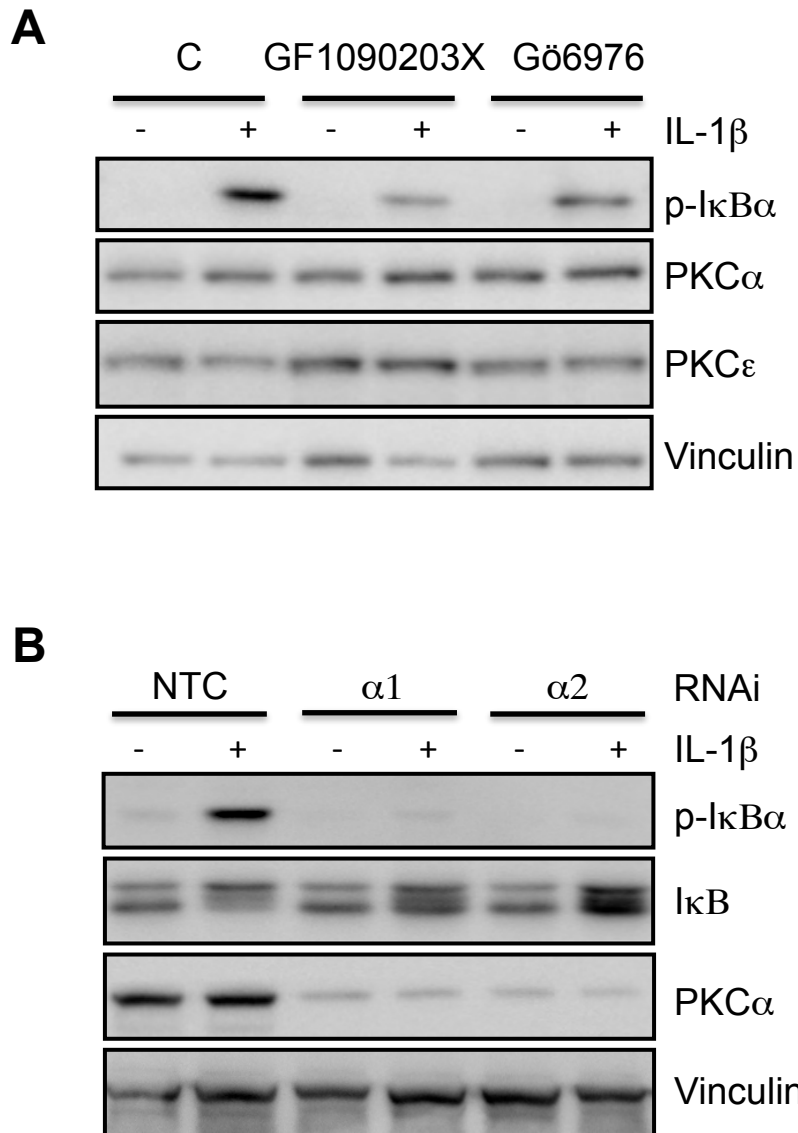
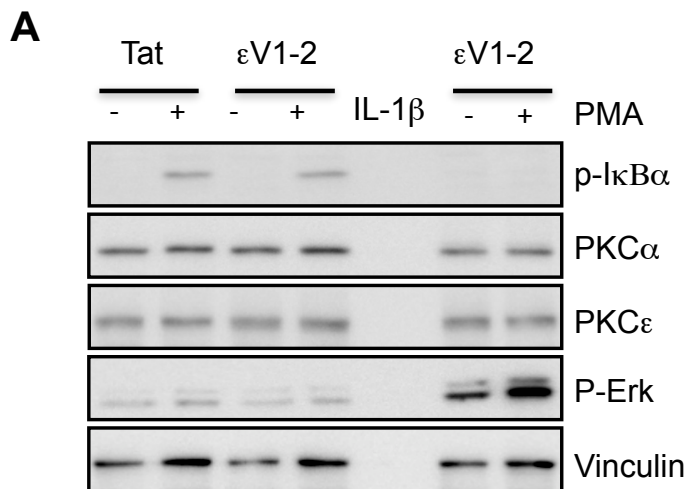
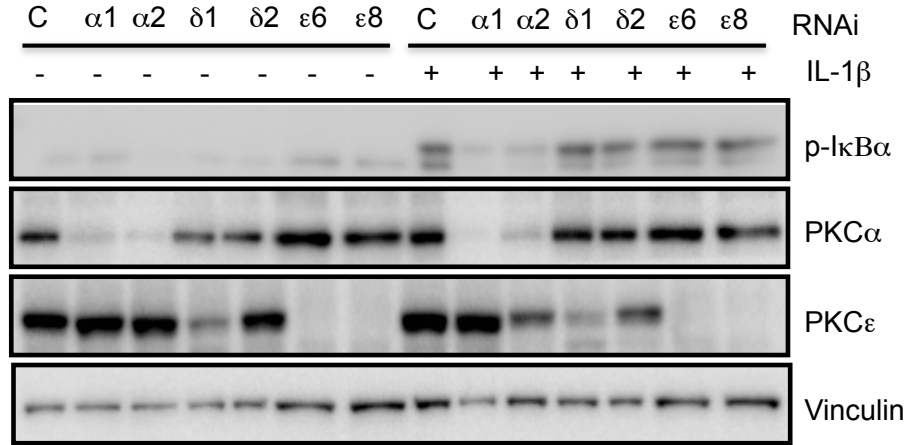
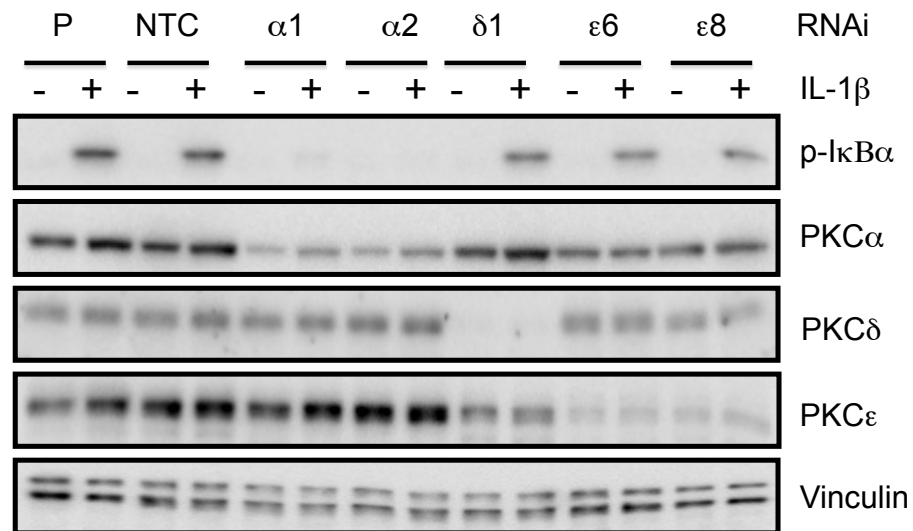


Figure 3.2 PKC α mediates phosphorylation of IkB α

(A) H460 cells were pre-treated with GF1090203X (5 μ M, 1 h) followed by treatment with IL-1 β (10 ng/mL, 5 min) and phosphorylated IkB α levels were determined by Western blot. (B) Cells were transfected with either PKC α RNAi duplexes ($\alpha 1$ or $\alpha 2$) or non-target control (NTC). After 48 h, cells were treated with IL-1 β (10 ng/mL) for 5 min and total and phosphorylated IkB α levels were determined by Western blot.

To specifically ascertain the role of PKC ϵ in IL-1 β -induced I κ B α phosphorylation, H460 cells were pre-treated with ϵ V1-2, a selective PKC ϵ inhibitor fused with the carrier peptide Tat, which blocks PKC ϵ translocation to membrane compartments and therefore prevents its activation. Contrary to the effect of Gö6976, ϵ V1-2 had no effect on phospho-I κ B α levels compared to the Tat peptide control (Fig. 3.3A). We also used RNAi for PKC ϵ and PKC δ to rule out the involvement of the nPKCs in the NF- κ B activation in H460 cells (Fig. 3.3B). Similar results were observed in H1975 cells (Fig. 3.3C). To further assess the role of PKC α in NF- κ B activation in response to IL-1 β , we took advantage of the NF- κ B luciferase reporter. As shown in Fig. 3.4, luciferase activity induced by IL-1 β was drastically reduced in PKC α -depleted H460 cells.



B**C****Figure 3.3 nPKCs do not mediate IL-1 β -induced I κ B α Phosphorylation**

(A) H460 cells were pre-treated either with the PKC ϵ specific inhibitor, $\epsilon v1-2$ (5 μ M, 1 h) or Tat carrier peptide and phosphorylation of I κ B was determined by Western blot. (B) Cells were transfected either with PKC α ($\alpha 1$ or $\alpha 2$), PKC ϵ ($\epsilon 6$ or $\epsilon 8$), PKC δ ($\delta 1$ or $\delta 2$) RNAi duplexes, or non-target control (NTC). After 48 h, cells were treated with IL-1 β (10 ng/mL) for 5 min and total and phosphorylated I κ B α levels were determined by Western blot. (C) The experiments in Fig 3.3B were repeated in H1975 cells.

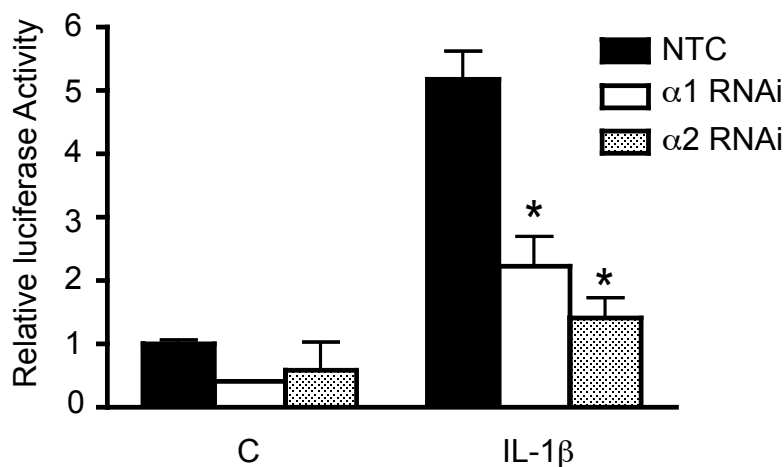


Figure 3.4 PKC α controls NF- κ B promoter activity

H460 cells were transfected with either PKC α RNAi duplexes ($\alpha 1$ or $\alpha 2$) or non-target control (NTC). 24 h after PKC α NTC or RNAi transfection, H460 cells were co-transfected with NF- κ B firefly luciferase reporter and pTK-*Renilla* plasmid. The following day, cells were stimulated with IL-1 β (10 ng/mL) and luciferase activity was determined after 6 h using the dual luciferase reporter assay.

PKC α is critical for NF- κ B nuclear translocation and DNA binding

Transcriptional regulation by NF- κ B also depends on its translocation from the cytosol to the nucleus following I κ B α degradation. As activation of the NF- κ B pathway leads to the relocalization of NF- κ B to the nucleus, we speculated that alteration of PKC α signaling may affect this NF- κ B intracellular mobilization. We used a cell fractionation approach to determine the effect of PKC α on IL-1 β induced NF- κ B nuclear translocation in H460 cells. As expected, IL-1 β promoted the translocation of NF- κ B from the cytosolic to nuclear compartments. This accumulation of NF- κ B in the nuclear fraction was completely blocked upon PKC α RNAi depletion (Fig. 3.5A). We then set out to identify whether IL-1 β -induced NF- κ B DNA binding involves PKC α signaling. To

address this issue we used electrophoretic mobility shift assay (EMSA) as an approach. As shown in Fig. 3.5B, stimulation with IL-1 β promotes an elevation in NF- κ B DNA binding activity in H460 cells nuclear extracts. Remarkably, this DNA binding activity was attenuated in nuclear extracts from PKC α -depleted H460 cells. Taken together these results confirm the requirement of PKC α in NF- κ B activation in response to IL-1 β in NSCLC cells.

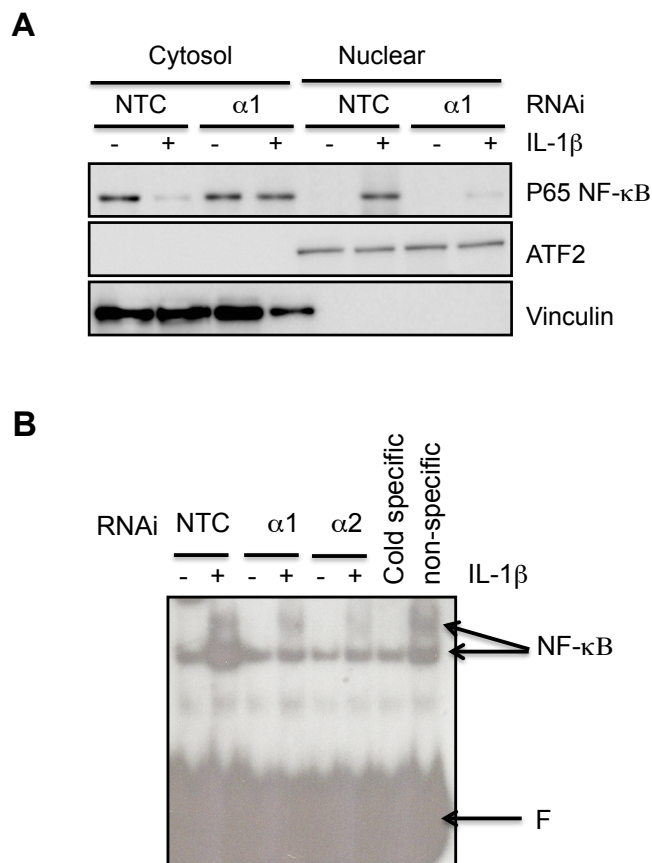
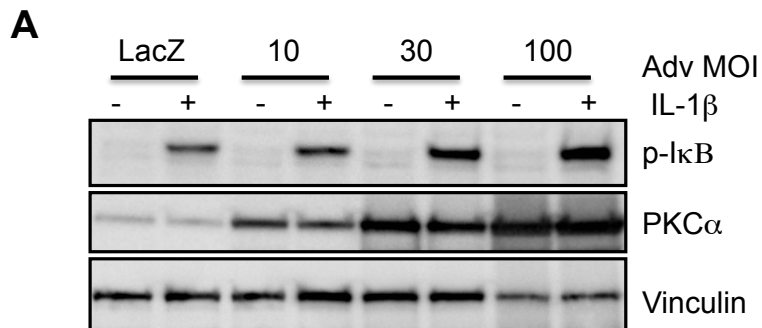


Figure 3.5 PKC α is critical for NF- κ B nuclear translocation and DNA binding

(A) Localization of NF- κ B was assessed in cytosolic and nuclear fractions after 1 h of IL-1 β (10 ng/mL) stimulation in control or PKC α depleted H460 cells using Western blot. ATF2 and vinculin were used as loading controls for nuclear and cytoplasmic fractions respectively. (B) The effect of PKC α depletion on NF- κ B DNA binding was measured using EMSA. The nuclear fractions were extracted 1 h after IL-1 β (10 ng/mL) stimulation or vehicle treatment. “F” represents free probe.

PKC α overexpression in normal lung epithelial cells enhances NF- κ B activation

As PKC α was found to play a significant role in IL-1 β -induced NF- κ B activation in lung cancer cells, we speculated that it could also have implications upon overexpression in normal lung epithelial cells. For these experiments, we used human bronchial epithelial cells (HBEC), a model of “normal” immortalized cells. HBEC cells were infected with different MOIs of a PKC α Adv, and LacZ Adv was used as a control. We observed that overexpression of PKC α potentiated IL-1 β induced phospho-I κ B levels (Fig. 3.6A). To further prove our hypothesis, we transfected PKC α overexpressing HBEC cells with the NF- κ B luciferase reporter as shown in Fig. 3.6B, overexpression of PKC α by itself enhanced NF- κ B luciferase activity. As a complementary approach, we measured NF- κ B DNA binding activity in response to IL-1 β in PKC α overexpressing HBEC cells. As expected, PKC α overexpression significantly increased NF- κ B DNA binding in response to IL-1 β (Fig. 3.6C).



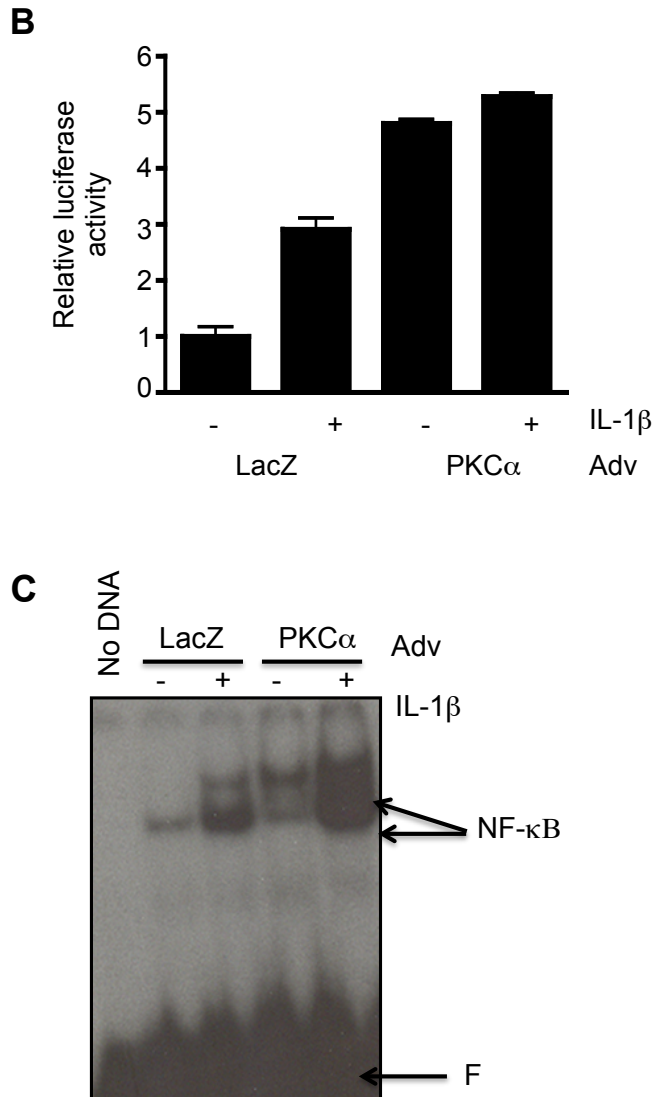


Figure 3.6 Overexpression of PKC α potentiates IL-1 β induced NF- κ B activation in normal lung epithelial cells

HBEC cells were infected with either LacZ Adv (100 MOI) or PKC α Adv at different MOI for 72 h and treated with IL-1 β (1 ng/mL, 5 min). (A) Protein levels of PKC α and phosphorylation of I κ B α were analyzed by Western blot after 5 min of IL-1 β stimulation. (B) PKC α overexpressing HBEC cells were co-transfected with NF- κ B firefly luciferase reporter and pTK-*Renilla* plasmid. 24 h later, cells were treated with IL-1 β (1 ng/mL, 6 h) and luciferase activity was determined. (C) Nuclear lysates of PKC α overexpressing HBEC cells treated with IL-1 β (1 ng/mL) for 5 min, were extracted and NF- κ B DNA binding was analyzed by EMSA.

PKC α regulates the expression of NF- κ B target genes in normal lung epithelial and cancer cells

Activation of NF- κ B is required for the induction of genes in response to IL-1 β . These genes are associated with survival and motility of lung cancer cells as well as production of a range of pro-inflammatory signals that contribute to chronic inflammation. Hence, to assess the functional relevance of PKC α signaling on NF- κ B transcriptional activity, we analyzed the effect of altering PKC α expression in both lung cancer and immortalized “normal” lung epithelial cells. Treatment of H460 cells with IL-1 β induces COX-2, IL-8 mRNA levels and MMP9 expressions. Interestingly, the induction in these NF- κ B regulated genes by IL-1 β was essentially abolished in PKC α -depleted H460 cells (Fig 3.7).

Next we overexpressed, we also overexpressed PKC α in HBEC cells using adenoviral approach. IL-1 β stimulation resulted in a marked elevation of COX-2 and MMP9 mRNA levels in HBEC. PKC α potentiated the induction of COX-2 and MMP9 in HBEC cells. For IL-8, IL-1 β caused a very high induction (~40-fold) in HBEC cell, and PKC α overexpression was unable to cause additional effect (Fig. 3.8).

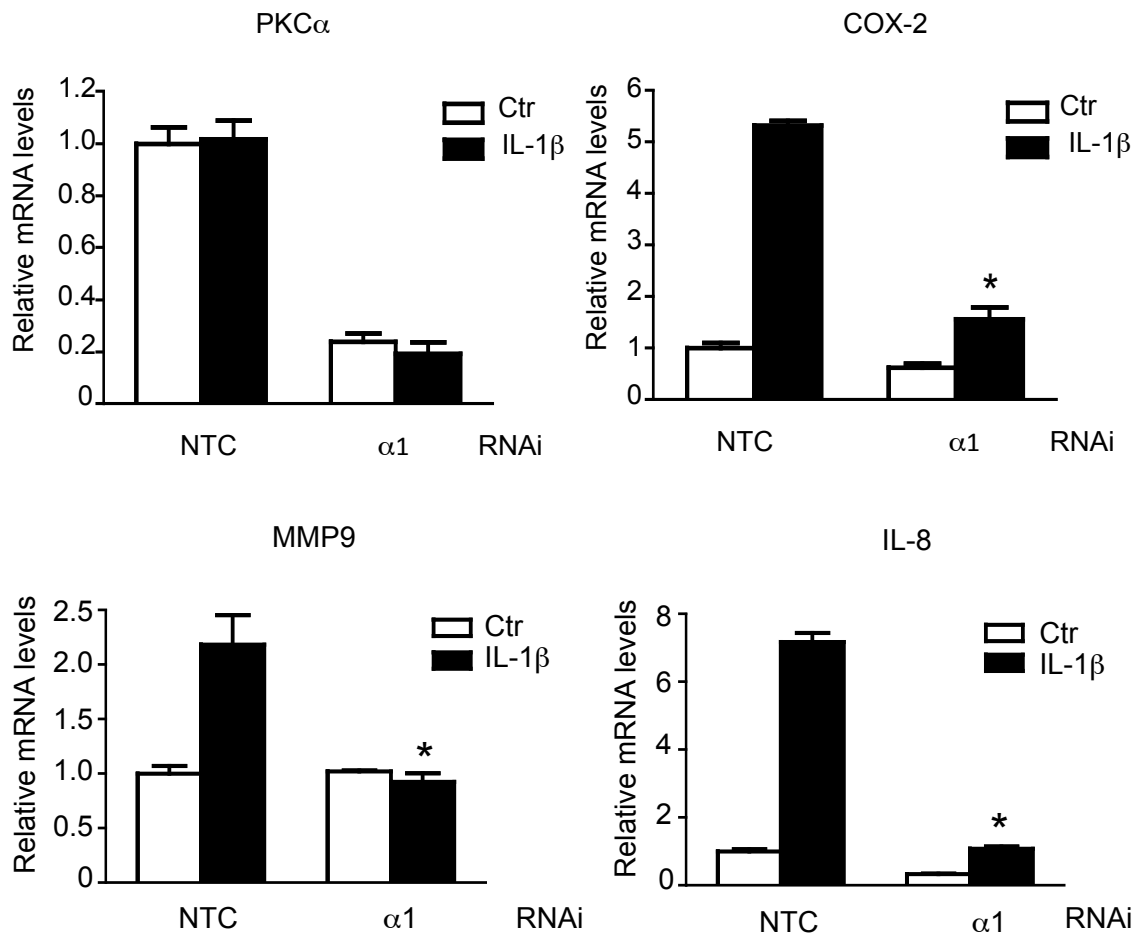


Figure 3.7 PKC α regulates expressions of NF- κ B target genes in NSCLC cells

H460 cells were transfected with either PKC α RNAi or NTC. After 48 h, cells were treated with IL-1 β (10 ng/ml) and RNA was isolated after 6 h. Gene expression of Cox-2, MMP9, and IL-8 was analyzed using qPCR.

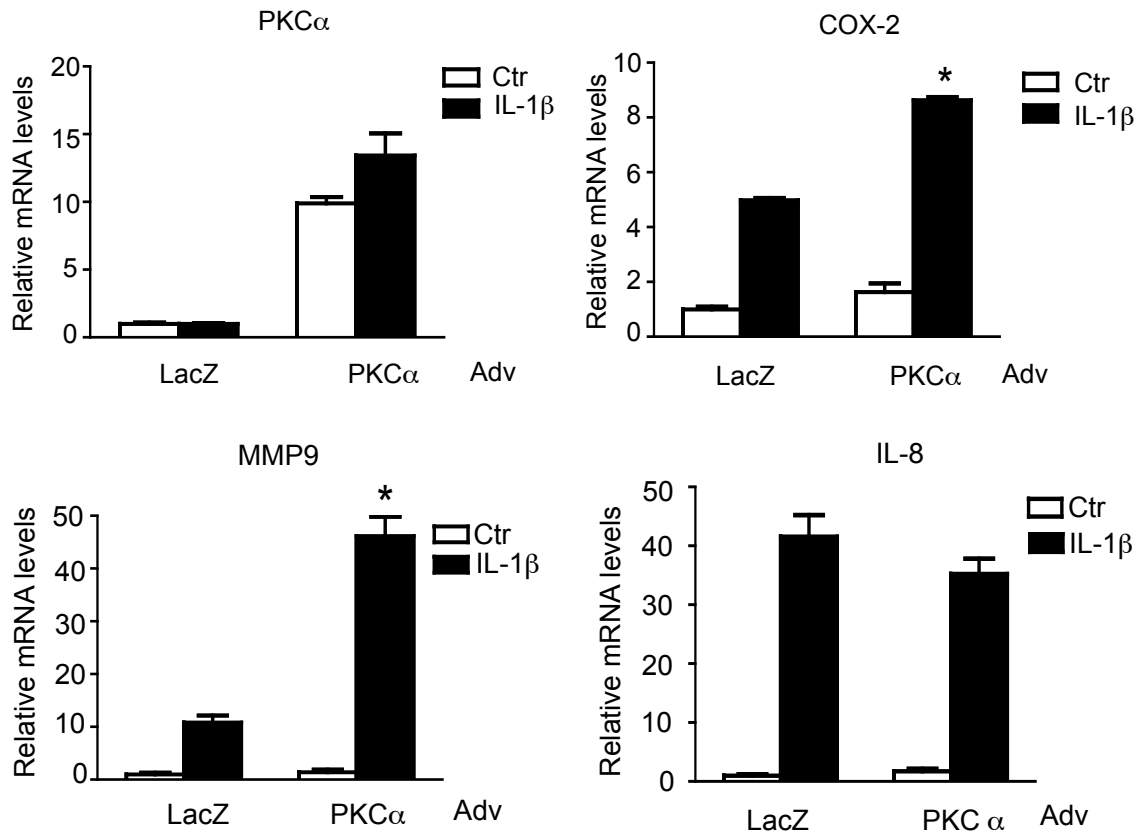


Figure 3.8 Overexpression of PKC α potentiates IL-1 β -induced expression of NF- κ B target genes in NSCLC cells

Normal lung epithelial (HBEC) cells were infected with either LacZ or PKC α Adv (100 MOI) for 72 h and stimulated with IL-1 β (10 ng/ ml) for 6 h. RNA was isolated and gene expression of Cox-2, MMP9, and IL-8 was analyzed using qPCR.

3.3 Discussion

There are ample reports implicating PKC isozymes in tumorigenesis. However, the specific contribution of individual PKC isozymes to various cellular processes that hallmark the initiation and progression of cancer remains only partially understood. This is also true for lung cancer, where PKC isozymes have been implicated in lung cancer malignancy, but their individual roles have not been yet fully characterized. In this study, we investigated whether PKCs are implicated in controlling the activation of the NF- κ B pathway in NSCLC. Our results show PKC α regulates the various steps required for NF- κ B activation in NSCLC cells. Notably, PKC α depletion impairs NF- κ B activation as well as induction of NF- κ B target genes in response to IL-1 β , arguing for an important role for PKC α in lung cancer progression.

The transcription factor NF- κ B plays an important role in host immune mechanisms. During inflammation, binding of pro-inflammatory cytokines such as TNF α or IL-1 β to their respective receptors results in activation of NF- κ B. Activation of NF- κ B is normally associated with acute inflammatory signals that involve chemokine-and adhesion molecule-mediated recruitment and activation of immune cells, mainly neutrophils to the site of inflammation. These acute inflammatory signals eliminate transformed cells and protect the body against cancer risks. However, during chronic inflammation, the role of NF- κ B becomes more complex and multifold [13, 71]. On one hand, continuous induction of pro-inflammatory factors by NF- κ B during chronic inflammation results in DNA damage, leading to oncogenic mutations. On the other hand, to withstand physiological stress caused by chronic inflammation, NF- κ B promotes

cell survival and proliferation by regulating the expression of proteins implicated in cell cycle control (*e.g.* cyclin D, c-myc) and apoptosis (*e.g.* IAP, Bcl-2), adding to its tumorigenic effects [109, 122].

The advanced lung cancer inflammation index (ALI) is used as a prognostic and treatment outcome marker for lung cancer patients, thus underscoring the significance of chronic inflammation in disease pathogenesis [84]. Chronic airway inflammation contributes to alterations in the bronchial epithelium and the lung microenvironment, paving a way for lung carcinogenesis. Additionally, lung cancer risk factors such as chronic obstructive pulmonary disease and chronic pulmonary infections are characterized by an inflammatory state. Lung tumors also exhibit high levels of inflammatory mediators such as ROS, cytokines, chemokines, and eicosanoids, which are associated with cancer initiation [67].

Cigarette smoke is the main cause of oxidative stress and etiological factor that induces lung cancer by generating ROS leading to tissue destruction. Inflammation due to smoking results in NF- κ B dependent up-regulation of pro-inflammatory cytokines including TNF α , IL-1 β , IL-8 and IL-6. Moreover, lung tumors and serum collected from lung cancer patients are reported to have elevated levels of inflammatory markers (C-reactive protein) and chemokines (such as CXCL13 and CCL22) that regulate migration of lymphocytes to the site of inflammation. Similarly, high levels of the p65 unit of NF- κ B were found in NSCLC compared to the normal lung epithelium [222]. Furthermore, IKK α/β independent activation of NF- κ B in response to growth factor receptors was found to be important for the survival of NSCLC cells and NF- κ B inhibition attenuated

the survival of lung cancer cells [29]. In addition to tobacco smoke, other environmental carcinogens have been causally linked NF- κ B activation in lung tumors. Urethane-induced lung tumor development was attenuated in mice with genetic depletion of the CCR5 chemokine. The CCR5^{-/-} mice were shown to have reduced NF- κ B activity compared to the wild type mice [98]. Other studies using mouse models have also shown the effect of constitutive NF- κ B activation in the development of pulmonary adenocarcinoma. Elevated NF- κ B activity was also reported in KRAS^{+/+};p53^{-/-} mice and KRAS-driven lung tumors were attenuated upon NF- κ B inhibition [10, 118].

Previous studies argued for a connection between PKC isozymes and the NF- κ B pathway activation. Initial links in cancer were reported for the α PKCs. Indeed, PKC ζ phosphorylates NF- κ B upstream kinase IKK β at ser-177 and ser-181 in response to TNF α . Additionally PKC ζ ^{-/-} mice have attenuated RelA binding activity [40]. The other α PKC isozyme, PKC ι also mediates phosphorylation of IKK β in response to TNF α and induces NF- κ B dependent transcription of the IL-6 gene in prostate cancer [106]. Recently, our laboratory identified a key role for PKC ϵ in TNF α -dependent activation of NF- κ B in prostate cancer. PKC ϵ was shown to be a part of the TNFRI signaling complex that leads to NF- κ B activation [54]. Yet other novel PKC isozyme, PKC δ was reported to facilitate RelA binding to the promoters of target genes [110]. Activation of the NF- κ B pathway is also attributed, in part, to alterations in cPKCs. PKC β -dependent activation of NF- κ B, through modulation of the IKK complex, was found to be crucial for the survival Chronic lymphocytic leukemia [111, 123]. Using *in vitro* binding assays, it has been

shown that PKC α forms a complex with the NF- κ B upstream kinase, IKK [192]. Additionally PKC α has been shown to interact with IKK β in response to PMA in 293T cells. The mechanism for PKC α -dependent NF- κ B activation was also extended to phosphorylation of RelA in glioma cells [126]. It has been reported that PKC α mediates NF- κ B activation in A549 NSCLC cells in response to TNF α [97].

IL-1 β has been shown to induce NF- κ B signaling in many cell types. Not surprisingly, we found that IL-1 β treatment activates the NF- κ B pathway in H460 cells as manifested by I κ B α phosphorylation and NF- κ B promoter activity. High levels of IL-1 β are found both in NSCLC cells and within the tumor microenvironment [167]. Additionally, polymorphisms in the IL-1 β gene have been associated with increased risk of NSCLC [223], an indication of the significance of IL-1 β -mediated signals in the pathogenesis of this disease. Here, we showed that depletion of PKC α prevents IL-1 β -induced NF- κ B activation as determined by decreased I κ B α phosphorylation, NF- κ B relocalization to the nucleus, NF- κ B promoter activity, and DNA binding. Studies using cellular and animal models have shown diverse implications of PKC α signaling in lung cancer. For example, PKC α up-regulates the cell cycle inhibitor p21 and confers a senescent phenotype in NSCLC cells [133]. In KRAS mutant mice, PKC α genetic silencing inhibited oncogene driven lung tumorigenesis [73]. However, there are also reports of PKC α as a positive regulator of growth and cell motility in NSCLC [31]. PKC α was shown to mediate ERK activation in the RAS signaling cascade [163]. Additionally, aprinocarsen, an antisense oligonucleotide against PKC α , has been used in

clinical trials for NSCLC patients. This antisense potentiates chemotherapeutic activity of carboplatin and paclitaxel in phase II trials although it did not enhance the overall survival of patients in phase III studies [137, 151]. In line with our results, Song *et al.* recently showed that inhibition of PKC α led to apoptosis in H460 NSCLC cells [165].

In contrast to the effects of PKC α , neither inhibition of PKC ϵ nor PKC δ significantly attenuated NF- κ B activation in NSCLC cells. These findings suggest that IL-1 β -dependent NF- κ B activation in H460 cells is highly dependent on PKC α . The role of PKC α in response to IL-1 β is not only limited to the cancer cells, but we also observed elevation of I κ B α phosphorylation and NF- κ B DNA binding in normal immortalized lung epithelial cells upon PKC α overexpression. The various biological effects of NF- κ B depend on its regulation of target gene expressions. Our findings show that PKC α -mediated activation of NF- κ B by IL-1 β results in up-regulation of NF- κ B responsive genes involved in cell survival, motility, angiogenesis and inflammation.

Summary

Our results have demonstrated that inhibition of PKC α controls the activation of the NF- κ B pathway. PKC α RNAi attenuates IL-1 β induced expression of NF- κ B responsive genes by decreasing NF- κ B transcriptional activity. Overall, these findings support the notion that distinctive PKCs play a central role in controlling the activation of the NF- κ B pathway.

3.4 Materials and Methods

Cell culture and reagents

The Human lung adenocarcinoma cell lines H460 and H1975 were obtained from ATTC (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10 % FBS (Hyclone, Logan, UT), 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Grand Island, NY). Immortalized human bronchial epithelial cells (HBEC) were a kind gift from Dr. Trevor Penning (University of Pennsylvania, PA) and were cultured in KSFM medium supplemented with 0.05 mg/mL bovine pituitary extract and 5 ng/mL EGF purchased from Life Technologies. Cells were maintained at 37°C in a humidified 5 % CO₂ atmosphere. IL-1β was purchased from R&D Systems (Minneapolis, MN) and used at 1 and 10 ng/mL concentrations. The NF-κB firefly luciferase reporter was a kind gift from Dr. Dave Manning (University of Pennsylvania, PA). PMA and the cPKC inhibitor Gö6976 were obtained from LC laboratories (Woburn, MA). The pan-PKC inhibitor GF109203X was procured from Enzo Life Sciences (Plymouth meeting, PA). The PKCε peptide inhibitor εV1-2 and the carrier peptide Tat were kind gifts from Dr. Daria Mochly-Rosen (Stanford University, CA).

Western blots

Western blot analysis was carried out as described on page 68. The following primary antibodies were used in 1:1000 dilutions: anti-PKCα (EMD Millipore Corp., Billerica, MA), anti-PKCε, anti-IκBα, NF-κB p65 (Santa Cruz Biotechnology, Santa

Cruz, CA), anti-PKC δ , anti-phospho-I κ B α (Cell Signaling Technology, Danvers, MA) and 1:50,000 dilutions of anti-vinculin (Sigma-Aldrich, St. Louis MO).

Adenoviral infections

Adenoviral infection was carried out as described on page 69.

RNA interference

RNA interference was carried out as described on page 69.

Luciferase Reporter Assay

Cells in 12-well plates (1×10^5 cells/well) were transfected with 0.45 μ g of a NF- κ B firefly luciferase reporter vector and 0.05 μ g of the *Renilla* luciferase expression vector pRL-TK (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen). The DNA/ Lipofectamine 2000 mixture was incubated for 20 minutes and added onto adherent cells in antibiotic free media. *Renilla* luciferase vector was used as an internal control to normalize the transfection efficiency. After 24 h, cells were treated with IL-1 β or vehicle and lysed 6 h later with passive lysis buffer (Promega). Luciferase activity was measured using the dual-luciferase reporter assay system. The results were expressed as a ratio of *Firefly* to *Renilla* luciferase activities in relative luciferase units (RLU).

Preparation of nuclear extracts

Cytoplasmic and nuclear extracts were obtained as described previously [155]. Briefly, cells were collected in ice-cold PBS and centrifuged to collect the cell pellets. Next, cells were resuspended in 250 μ l of hypotonic buffer (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 10 % glycerol, 0.2 % Igepal and protease inhibitors) for 10 min. Lysed cells were centrifuged ($2000 \times g$) for 1 min and the supernatant containing the cytoplasmic extract was removed. Incubation in hypotonic buffer and centrifugations were repeated three times to clean the nuclear pellet. The pellet was then resuspended in 100 μ l hypertonic buffer (240 mM NaCl, 20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 20 % glycerol, 0.2 % Igepal, and protease inhibitors) and incubated for 30 min at 4°C. After 15 min centrifugation ($12000 \times g$), the supernatant (nuclear extract) was collected. Both cytoplasmic and nuclear fractions were stored at -80°C until use.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as described previously [54]. Briefly, an NF- κ B oligonucleotide probe (5'-agcttGAGGGGATTCCCTTA-3') was labeled with [α - 32 P] dATP using Klenow enzyme. Following purification on a Sephadex G-25 column, the binding reaction was carried out at 25 °C for 10 min in two sets. The first set contained 5 μ g of nuclear proteins, 1 μ g of poly (dI-dC), and 10^6 cpm of labeled probe in a final volume of 20 μ l of binding buffer (10X buffer: 100 mM Tris-HCl pH 7.5, 500 mM NaCl, 50 mM MgCl₂, 100 mM EDTA, 10 mM DTT, 1 % Triton X-100, and 50 % glycerol), while the second set had no nuclear extracts. To show a binding specificity, 50-fold molar

excess of cold NF- κ B probe and cold AP-1 probe were used. In the reaction, DNA-protein complexes and unbound probe were separated electrophoretically on 6 % non-denaturing polyacrylamide gels in 0.5X TAE buffer at 200 V. The gel was dried, followed by autoradiography at -80°C.

RNA isolation and DNA synthesis

RNA isolation and DNA synthesis was carried out as described on page 68.

Real-time PCR

Quantitative real time PCR was carried out as described on page 68. Primers and fluorogenic probes for PKC α , COX-2, IL-8, MMP9 and 18S were purchased from Applied Biosystems.

Statistical Analysis

All statistical analyses were done using GraphPad Prism v5.03 (GraphPad Software, Inc., San Diego, CA). A *p* value < 0.05 was considered statistically significant.

Chapter 4: PKC ϵ is required for KRAS-driven lung tumorigenesis

4.1 Introduction

KRAS mutations have been identified in approximately 30 % of human lung adenocarcinomas [41, 87]. *KRAS* is a member of the RAS small GTPase superfamily and functions to propagate mitogenic and survival signaling through activation of the MAPK cascade and PI3K pathway [87]. Thus, *RAS* mutations result in uncontrolled stimulation of these signaling pathways and contribute to tumorigenesis. *KRAS* mutations are detected in precancerous lesions of lung adenocarcinoma suggesting their role in the initiation of human lung cancer. Similarly, *KRAS* mutations were the first molecular alterations identified in mouse lung tumors and mutation of *KRAS* is a major early event in the carcinogenic process of both spontaneous and chemically-induced mouse lung tumors [83, 202], suggesting that mouse lung tumorigenesis may at least partly model human lung cancer [129]. Several distinct mouse models that express mutant *KRAS* at physiologically normal levels have been generated and show that mutation in this oncogene is sufficient to drive proliferation and induce the formation of lung adenocarcinomas [42, 117, 129]. One of these models is the LSL-*KRAS*^{G12D} mice, in which expression of oncogenic *KRAS*^{G12D} is expressed in the mouse lung upon delivery of Cre-recombinase. This leads to the development of lung lesions, ranging from atypical adenomatous hyperplasia to adenocarcinomas. This animal model thus recapitulates the stages of tumor progression found in NSCLC patients [42].

PKC ϵ is an oncogenic kinase that is dysregulated in many cancers including NSCLC. Overexpression of PKC ϵ is reported in more than 90 % of primary NSCLC cancers relative to the normal lung epithelium [7]. Several studies from our laboratory

underscored the importance of PKC ϵ in lung cancer progression. We previously reported that PKC ϵ regulates NSCLC cell proliferation, growth and tumorigenicity in nude mice. Indeed, PKC ϵ RNAi depletion from NSCLC cells reduces anchorage-dependent and anchorage-independent growth in H358 and A549 cells. Moreover, NSCLC growth in xenografts in nude mice is substantially impaired by PKC ϵ RNAi depletion or treatment with the specific PKC ϵ inhibitor ϵ V1-2 [25]. Additionally, our lab has shown that PKC ϵ is required for the activation of the small GTPase Rac1 and in this way it controls NSCLC cell motility and invasiveness through regulating the secretion of metalloproteinases [26]. PKC ϵ also regulates the expression of anti-apoptotic genes such as Bcl-2 and IAP-2 [12, 63] and controls G1 to S transition in the cell cycle [19, 49]. Additionally, it was previously reported that PKC ϵ depletion sensitizes lung cancer cells to TRAIL-induced cell death [47].

A growing body of evidence indicates that PKC ϵ is implicated in the initiation and maintenance of cancer phenotype. Early studies revealed that overexpression of PKC ϵ in fibroblasts and colonic epithelial cells leads to malignant transformation, which includes morphological changes, anchorage-independent growth, and increased tumorigenesis in nude mice [23, 119, 177]. Overexpression of PKC ϵ was found to transform androgen-dependent prostate cancer cells (LNCaP) to androgen-independent cancer cells [206]. Additionally, our laboratory showed that overexpression of PKC ϵ in the mouse prostate is sufficient to induce preneoplastic lesions [18], as well as it cooperates with PTEN loss to induce prostate adenocarcinoma (unpublished results). In skin tumor animal models, PKC ϵ overexpression also resulted in hyperplasia,

hyperkeratosis and the development of skin squamous cell carcinoma in mice treated with DMBA and a phorbol ester [6]. Thus, it is conceivable that PKC ϵ is implicated in tumor initiation in lung cancer.

Several studies implicated PKC ϵ in RAS signaling in cancer. PKC ϵ can be activated by KRAS and is required for RAF-induced cell transformation. Inhibition of PKC ϵ attenuated NIH 3T3 and Cos-1 cell proliferation similar to dominant negative RAS and RAF. Moreover, it has been shown that PKC ϵ binds to the RAF kinase domain and activates its kinase activity by direct phosphorylation [5, 23]. There have been reports suggesting a link between PKC ϵ and chemically-induced lung carcinogenesis. Indeed, PKC ϵ depletion was found to suppress cell growth in response to tobacco smoke. At the mechanistic level, activation of PKC ϵ by ROS generated from tobacco smoke recruits and phosphorylates the metalloprotease TACE/ADAM-17. Activation of TACE results in the cleavage of amphiregulin, leading to transactivation of EGFR [100]. PKC ϵ has been further implicated in the induction of nitric oxide synthase (iNOS), predisposing smokers to lung cancer [131]. However, a link between PKC ϵ and KRAS tumorigenesis *in vivo* still remains to be established.

Here, we examined the requirement for PKC ϵ in lung tumorigenesis *in vivo* by using a mouse model. We found that genetic inhibition of PKC ϵ significantly attenuates KRAS-driven initiation of lung tumors. Additionally, we initiated the generation of an inducible lung specific PKC ϵ mouse model to study if the overexpression of PKC ϵ is sufficient to promote the formation of preneoplastic or neoplastic lesions.

4.2 Results

Generation of $KRAS^{G12D}; PKC\epsilon^{-/-}$ mice

Oncogenic KRAS-driven mouse models of lung adenocarcinoma have enabled the study of tumor initiation and progression *in vivo*. In order to determine whether PKC ϵ cooperates with KRAS to promote lung tumor formation, we created a mouse model in which mutated active KRAS was conditionally expressed in PKC ϵ heterozygous or knockout backgrounds. Previous studies have reported that PKC ϵ knockout mice display decreased tolerance to alcohol, delayed pain signals and reduced acute cardio protection, but other than that there are no obvious general abnormalities. Experimental mice were developed by crossing mice harboring a conditional activated *KRAS* allele (*LSL-KRAS^{G12D}*) with PKC ϵ knockout mice. To achieve the PKC ϵ knockout background, we first interbred the PKC $\epsilon^{-/+}$ progeny. In the KRAS model, mutant is expressed as heterozygous whereas homozygotes die in utero [42]. Spontaneous lung tumor initiation is achieved by intranasal delivery of Cre-recombinase to the lungs using an adenovirus (Ad-Cre). This leads to removal of the transcriptional stop element and expression of the oncogenic KRAS. To generate the bitransgenic $KRAS^{G12D};PKC\epsilon^{-/-}$ mice, we used two approaches. The strategies involved crossing $KRAS^{G12D};PKC\epsilon^{+/-}$ mice with $PKC\epsilon^{-/-}$ mice, and $KRAS^{G12D};PKC\epsilon^{+/-}$ mice with $PKC\epsilon^{+/-}$ mice, which had 25 % and 12.5 % chance of achieving the experimental mice, respectively (Fig. 4.1). The bitransgenic mice appeared normal at birth and did not display any signs of abnormalities during adulthood. The deletion of *PKC ϵ* gene (*PRKCE*) and the expression of mutant *KRAS* were verified using qPCR (Fig. 4.2A and 4.2B).

First generation

$PKC^{\varepsilon-/-} \times KRAS^{G12D}$

Parents	allele	Combination
Father	P-P- K+K+	P-K+
Mother	P+P+K+K-	P+K+, P+K-

F1	P+K+	P+K-
P-K+	P-/+K+/+	P-/+K+/- (50 %)

Second generation

Strategy 1

$PKC^{\varepsilon-/-} \times KRAS^{G12D}; PKC^{\varepsilon-/+}$ (F1)

F2	P-K+
P+K+	P+/-K+/+
P+K-	P+/-K-/+
P-K+	P-/-K+/+
P-K-	P-/-K-/+

Strategy 2

$PKC^{\varepsilon-/+} \times KRAS^{G12D}; PKC^{\varepsilon-/+}$ (F1)

F2	P+K+	P-K+
P+K+	P+/+K+/+	P+/-K+/+
P+K-	P+/+K-/+	P+/-K-/+
P-K+	P-/+K+/+	P-/-K+/+
P-K-	P-/+K-/+	P-/-K-/+

Figure 4.1 Breeding strategies to generate the $KRAS^{G12D}; PKC^{\varepsilon-/-}$ mice

PKC ε knockout mice were crossbred with KRAS mutant mice. The F1 progeny were crossed either with PKC ε heterozygous or PKC ε knockout mice to generate the KRAS mutant in PKC ε null background.

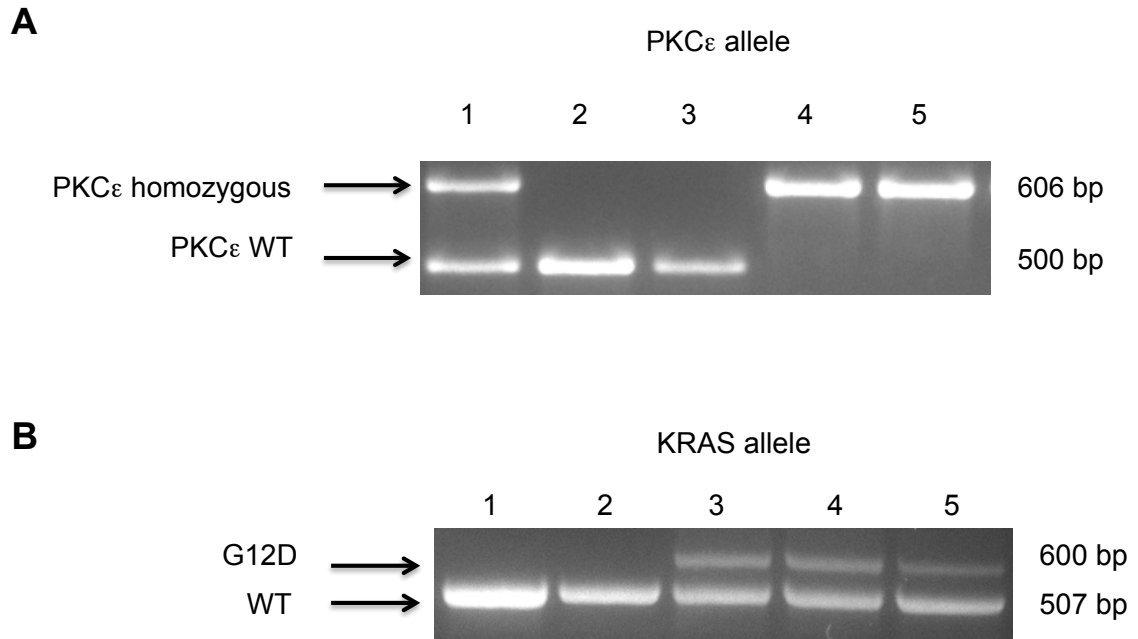


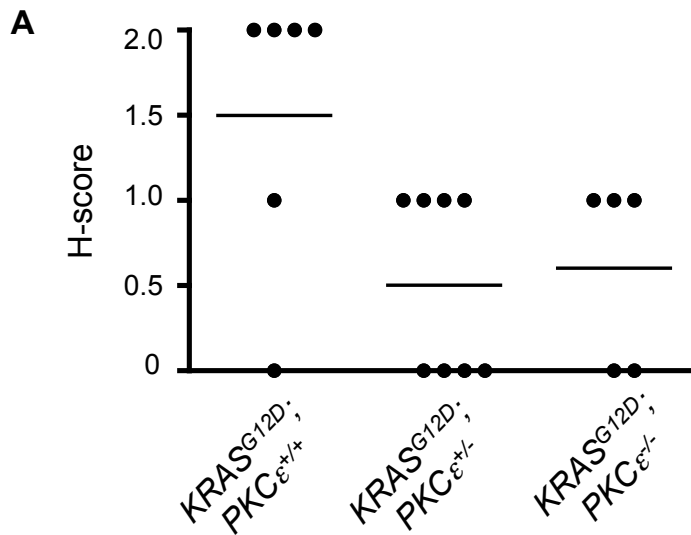
Figure 4.2 *KRAS*^{G12D} and *PKCε* mutant mice genotyping

(A) Genomic DNA was extracted and status of wild-type, heterozygous or homozygous PKC ϵ allele was assessed by PCR. (B) Status of wild-type or mutant KRAS allele was assessed by PCR.

Genetic ablation of PKC ϵ attenuates tumor formation by KRAS

Given the observation that PKC ϵ and KRAS are dysregulated in a large proportion of lung tumors, we asked whether loss of PKC ϵ expression affects the formation of KRAS-driven tumors. It has been reported that 12 to 16 weeks following Ad-Cre administration, KRAS mice develop adenocarcinomas and die within 24 to 26 weeks after activation of the oncogene [42, 83, 92]. To evaluate the effects of PKC ϵ deletion *in vivo*, we randomized mice into three groups; *KRAS*^{G12D};*PKCε*^{+/+},

$KRAS^{G12D};PKC\epsilon^{+/-}$ and $KRAS^{G12D};PKC\epsilon^{-/-}$. Mice were exposed to Ad-Cre at 6 to 8 weeks-old. Interestingly, KRAS-driven tumor formation in either PKC ϵ heterozygous or PKC ϵ knockout mice was significantly reduced compared to KRAS mutant mice with PKC ϵ wild-type background. Therefore, loss of one PKC ϵ allele was sufficient to significantly affect lung tumor initiation (Fig. 4.3A). Neither the $KRAS^{G12D};PKC\epsilon^{+/-}$ nor the $KRAS^{G12D};PKC\epsilon^{-/-}$ mice had incidence of extensive tumors. Indeed, 83.3 % of the $KRAS^{G12D};PKC\epsilon^{+/+}$ mice developed extensive tumors, whereas only 53.8 % of the $KRAS^{G12D};PKC\epsilon^{+/-}$ or $KRAS^{G12D};PKC\epsilon^{-/-}$ mice had small patchy areas of tumors. Histological analysis also revealed that while $KRAS^{G12D};PKC\epsilon^{+/+}$ mice have multifocal lung lesions, the lesions were markedly reduced in PKC ϵ heterozygous mice and nearly absent in a PKC ϵ -null background (Fig. 4.3B).



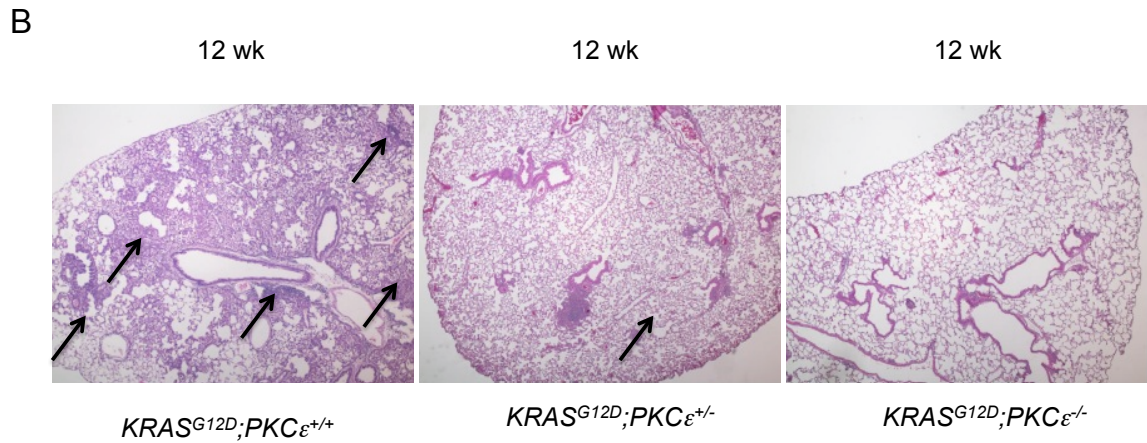


Figure 4.3 Genetic inhibition of PKC ϵ impairs lung tumorigenesis by mutant KRAS

(A) Histological scoring system for lung tumors formed in the bitransgenic $KRAS^{G12D}; PKC\epsilon^{-/+}$ and $KRAS^{G12D}; PKC\epsilon^{-/-}$ or $KRAS^{G12D}$ mice. 0 = minimal tumor seen; 1 = small patchy areas of tumors; 2 = extensive lung tumors. (B) Representative histological sections of $KRAS^{G12D}$, $KRAS^{G12D}; PKC\epsilon^{-/+}$ and $KRAS^{G12D}; PKC\epsilon^{-/-}$ lungs 12 weeks postinfection with Ad-Cre (5×10^5 PFU).

Generation of *pTRE-PKCε* Mice

We have previously shown that PKC ϵ is required for NSCLC cells growth and tumorigenicity in nude mice [25, 26]. Studies have indicated that there is a causal relationship between up-regulation of PKC ϵ and cancer initiation in prostate and skin cancer [6, 18]. We sought to investigate the phenotypic consequence of overexpressing PKC ϵ in the normal lung *in vivo*. As an approach we generated a transgenic mouse model in which PKC ϵ was specifically expressed in the lungs. In an inducible manner, PKC ϵ cDNA was inserted into a modified pTRE-tight promoter vector to generate a cassette which contained the tetracycline-responsive element (TRE), minimal CMV promoter, N-terminal HA-tag and the PKC ϵ gene. This construct allows turning transgene expression “on” and “off” by doxycycline treatment. To validate the expression of PKC ϵ , COS-1 cells were transiently co-transfected with pTRE-PKC ϵ and pTetON plasmid. 24 h later, cells were treated with increasing concentrations of doxycycline. We found a concentration dependent elevation in PKC ϵ expression in COS-1 cells (Fig. 4.4A). The results were also further confirmed in MCF-7 cells that stably express the reverse transcription transactivator (rtTA). In the presence of doxycycline, rtTA enabled the expression of PKC ϵ upon binding to the Tet-responsive element (Fig. 4.4B). Following *in vitro* validations, linearized plasmid was microinjected into the pronucleus of fertilized zygotes of S129/B6 mice and implanted into a pseudo-pregnant foster mother. Chimeric mice were analyzed by Southern blot to select founders for transgenic lines. To detect the PKC ϵ transgene, an endogenous PKC ϵ probe was used. As shown in Fig. 4.5, an

additional band was detected in DNA samples from the PKC ϵ transgenic mice. This band was absent in samples from wild-type C57BL/6 or mice that lack the transgene insertion.

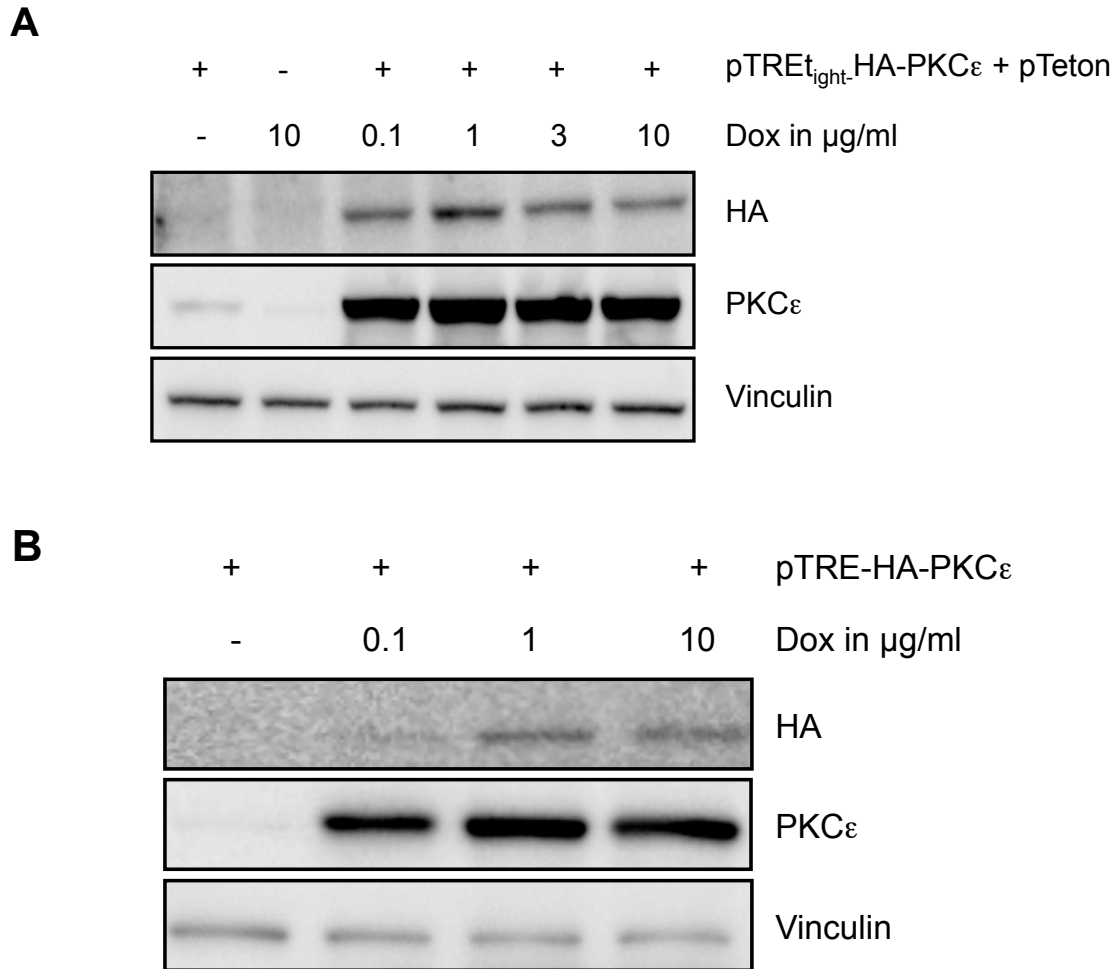


Figure 4.4 Validation of *pTRE-PKC ϵ* vector construct before pronuclear microinjection

(A) Cos-1 cells were co-transfected with pTet-On and pTRE-PKC ϵ . The expression of PKC ϵ under the control of a tetracycline (*Tet*)-inducible promoter in response to different concentrations of doxycycline was verified by Western blot. (B) MCF-7 Tet-On cells were transfected with pTRE-PKC ϵ and the expression of PKC ϵ upon doxycycline treatment was detected by Western blot.

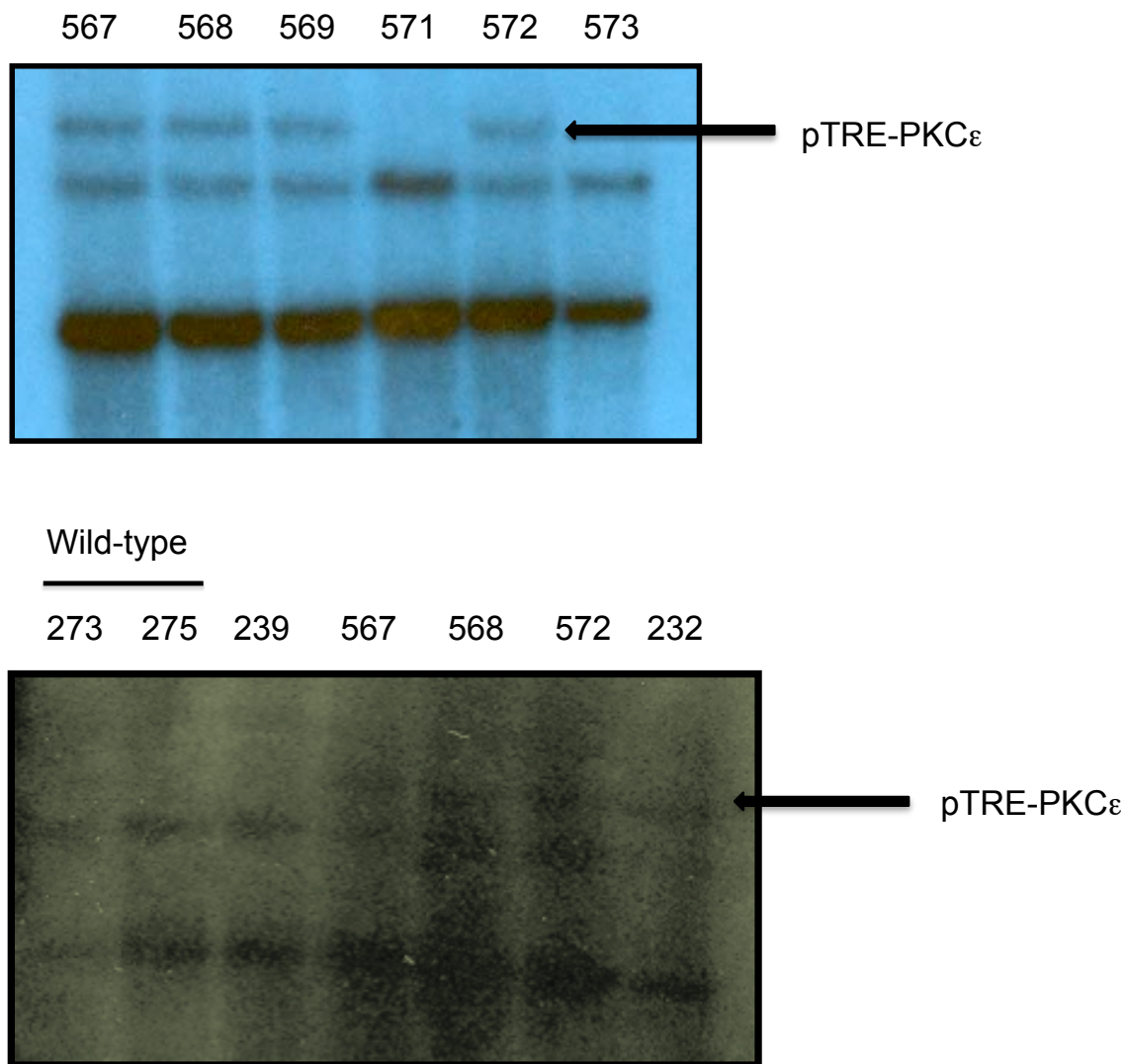


Figure 4.5 Identification of transgenic founder lines

Genomic DNA was extracted from two wild-type and seven *pTRE-PKC ϵ* transgenic mice and digested overnight with *Bgl*III restriction enzyme. An endogenous PKC ϵ sequence was used as a probe and the number of pTRE-PKC ϵ integrations in the genome was assessed by Southern blot.

4.3 Discussion

PKC ϵ as a mediator of KRAS tumorigenesis

KRAS is a common mutation in many cancers, and the identification of mediators of KRAS signaling is important to successfully develop therapeutic approaches. While the oncogenic role of *KRAS* gene is widely established, the molecular effectors responsible for its effects in lung cancer have not yet been fully elucidated. Here we found that PKC ϵ is required for KRAS-driven tumorigenesis in lungs. Genetic loss of PKC ϵ results in attenuated tumor formation upon activation of oncogenic KRAS in mouse lungs. Notably, loss of one allele was sufficient to significantly inhibit KRAS-dependent lung tumorigenesis.

Lung-specific KRAS mutant mouse models have been widely used for understanding the biology associated with KRAS-induced lung carcinogenesis. Three different strategies have been previously used to express mutant KRAS for inducing lung tumors *in vivo*. First, expression of a doxycycline-regulated KRAS^{G12D} transgene was achieved using transgenic mice expressing reverse tetracycline transactivator transgene under the control of surfactant factor C (SPC) promoter. In this model, tumor formation is induced by doxycycline and the expression of KRAS^{G12D} can be turned “on” and “off” at different time points [104]. The second strategy involves somatic recombination that results in expression of KRAS^{G12D}. Although most tumors are formed in the lungs, studies also reported tumors in other tissues [117]. The third model, LSL-KRAS^{G12D}, which is the model we used in our studies, involves the expression of mutant KRAS upon Cre-mediated removal of a stop codon sequence flanked by loxP. Cre-recombinase is

delivered to the lungs either by an infection with adenoviral vector or by crossbreeding with mice that express Cre under the control of an SPC promoter. All three models ultimately result in the formation of lung tumors [83].

Using LSL-KRAS^{G12D} mice, several studies have identified downstream effectors of KRAS in lung tumorigenesis. For example, deletion of NOS2 attenuated lung tumor formation in mice expressing oncogenic KRAS [131]. The small GTPase Rac1 has also been implicated in KRAS-induced lung cancer. *KRAS^{G12D};Rac1^{-/-}* mice showed delayed tumor initiation, and the tumor to lung volume ratio was significantly reduced compared to the Rac1 wild-type control mice [92]. Our studies suggest that PKCε is another key effector in the initiation of KRAS-driven lung tumors. Twelve weeks after Ad-Cre infection, KRAS mutant mice with PKCε heterozygous or knockout background had reduced tumor incidence compared to *KRAS^{G12D};PKC^{+/+}* mice. These results are consistent with a tumor promoting role of PKCε in primary human NSCLC tumors. Indeed, previous studies from our laboratory revealed that RNAi depletion of PKCε from NSCLC cells results in significant inhibition of anchorage-dependent and independent growth. Moreover, silencing PKCε in KRAS mutant NSCLC cells, such as A549, H441, H358 and H322 cells reduced growth, proliferation and resulted in decreased metastatic dissemination in xenograft models [25].

Our results raise a number of interesting questions for subsequent work, including what role PKCε plays in maintenance of KRAS-induced lung tumors, as well as what mechanisms and downstream effectors of PKCε mediate KRAS signaling. To establish the important role of PKCε in tumor maintenance, we have initiated a survival

experiment. In this experiment, 6-8 weeks old $KRAS^{G12D};PKC\epsilon^{+/+}$ and $KRAS^{G12D};PKC\epsilon^{+/-}$ mice were infected with Ad-Cre, and mice are being observed until they require euthanasia due to tumor burden. We expect differential survival rates between mice with PKC ϵ wild-type and heterozygous backgrounds. Due to the tumorigenic role of PKC ϵ in lung cancer, the $KRAS^{G12D};PKC\epsilon^{+/-}$ mice are expected to live longer. However, we can only speculate on the tumor burden differences at the time of death between the two groups. In a study that analyzed the role of Rac1 using similar KRAS-driven mouse lung tumor models, Rac1 was shown to delay tumor initiation. Additionally, KRAS mutant mice in a Rac1 knockout background survived longer post-infection with Ad-Cre [92]. In the $KRAS^{G12D};NOS2^{-/-}$ model, mice had increased tumor latency, decreased tumor growth, and longer survival time compared to the $KRAS^{G12D}$ mice in $NOS2^{+/+}$ background. At the time of death, NOS2 wild-type mice had 100 % incidence of at least one carcinoma, while the incidence was slightly reduced to 89.5 % in NOS2 knockout background [131]. Since PKC ϵ has been implicated in the regulation of both Rac1 and NOS2 in lung cancer and also has tumorigenic capacity in NSCLC cells *in vitro*, we expect that $KRAS^{G12D}; PKC\epsilon^{-/-}$ mice, will display significant decrease in tumor size. Several other effectors of PKC ϵ could mediate its tumorigenic capacity. Studies from our laboratory revealed that lesions that developed in mice overexpressing PKC ϵ in the prostate exhibited elevated Akt activation [18]. Additionally, depletion of Akt in mice that harbor a latent activatable mutant KRAS ($KRAS^{LA2/+}$) resulted in reduced lung tumor formation. Akt has also been implicated in chemically-induced lung carcinogenesis [76, 89]. The Erk/MAPK cascade and the Stat3 pathways are other potential mechanisms by

which PKC ϵ could drive tumor formation in the lungs as both are well-established PKC ϵ effectors in other models [5].

Inducible model for PKC ϵ in mouse lungs

We aimed to inducibly overexpress PKC ϵ in the lungs to recapitulate the scenario observed in cancer patients. Additionally, by turning off the overexpression of PKC ϵ , we can determine if the kinase is important for the maintenance of lung tumors. The pTRE-PKC ϵ mouse model we generated will hopefully result in the overexpression of PKC ϵ specifically in the lung when crossed with SPC-rtTA transgenic mice. SPC is exclusively expressed in type II alveolar epithelial cells, which are sites of lung cancer occurrence. The bitransgenic mice, SPC-rtTA-PKC ϵ mice will express the reverse tetracycline controlled transactivator (rtTA) protein under the control of the human SPC promoter. rtTA then binds to the promoter of the tetracycline element (pTRE) and drives the expression of PKC ϵ in the presence of doxycycline [42, 83]. An additional advantage of the pTRE-PKC ϵ mice is that they can be used to generate other tissue-specific transgenic models by crossing with appropriate mouse models that direct transgene expression in different cell types.

As we are currently optimizing the PCR assay for genotyping the pTRE-PKC ϵ mice, we can only speculate about the phenotypic outcome of the SPC-rtTA-PKC ϵ mice at this stage. Previously our lab has generated transgenic mice that overexpress PKC ϵ in the mouse prostate under the control of the probasin promoter. Overexpression of PKC ϵ in these transgenic mice led to the development of preneoplastic lesions, which

progressed to invasive adenocarcinoma of the prostate in a PTEN-deficient background. Similar results are expected from the lung-specific PKC ϵ mice where overexpression of PKC ϵ may lead to precancerous lesions of the lung. To develop adenocarcinoma of the lungs, PKC ϵ may require cooperation from other genetic alterations such as KRAS or EGFR mutations. Similarly, the requirement of carcinogens for PKC ϵ -dependent tumor progression from hyperplasia to carcinoma was seen in skin-specific PKC ϵ overexpressing mice. Although these mice developed hyperplasia, squamous cell carcinoma was induced 12 weeks after combinational treatment with DMBA and a phorbol ester [6].

Summary

Here we have presented evidence showing the requirement of PKC ϵ for KRAS induced mouse lung tumors using a bitransgenic *KRAS^{G12D};PKC ϵ ^{-/-}* mouse model generated in our lab. We have also generated a pTRE-PKC ϵ mouse model that can be used for lung-specific overexpression of PKC ϵ in an inducible manner. These animal models will enable us to elucidate the diverse roles PKC ϵ plays in the initiation and maintenance of lung tumors. Additionally, the lung-specific PKC ϵ overexpressing mice will enable us to delineate oncogenic alteration that cooperate with PKC ϵ to promote lung tumors *in vivo*.

4.4 Material and Methods

Mice Strains

PKCε^{-/+} mice were developed in the laboratory of Dr. Robert Messing (UCSF) as described previously [91]. Briefly, a mouse genomic *PKCε* sequence with depletion of the start codon was inserted to a recombination vector. The linearized construct was electroporated into embryonic stem cells. Clones were selected in culture in the presence of hygromycin and neomycin and analyzed for predicted homologous integration. Recombinant clones were then microinjected into blastocysts and implanted in pseudo-fertilized mice. The chimeric mice were crossed with *C57BL/6* WT mice. *LSL-Kras*^{G12D} heterozygous mice (B6.129-*Kras*^{tm4Tyj}), originally developed by Dr. Tyler Jacks, was obtained from Jackson laboratories. Briefly, the G12D mutation was inserted in exon 1 of the *KRAS* gene followed by insertion of loxP-flanked stop element in intron 1, upstream of the mutation. The linearized vector was electroporated into embryonic stem cells. The chimeric mice were backcrossed to *C57BL/6* for more than 10 generations by The Jackson Laboratory.

KRAS^{G12D} and *PKCε* mutant mice genotyping

To genotype mice, genomic DNA was extracted from tail clippings and analyzed by PCR using the Hot Start PCR kit from New England Biolabs. The sequence of PCR primers for confirmation of *KRAS*^{G12D} and heterozygous or knockout *PKCε* backgrounds were provided from the Jackson Laboratory and the Messing laboratory respectively. PCR primers for confirmation of *KRAS*^{G12D} were 5'-GTCGACAAGCTCATGCGGGTG,

5'-CCTT TACAAGCGCACGCAGACTGTAG and 5'-
AGCTAGCCACCATGGCTTGAGTA AGTCTGCA. For *PKCε* the primers were 5'-
ACATGCAGAATGAGTACCGG-3', 5'-TCAACATCTCCTGGTGGAAAC-3' and 5'-
AATATGCGAAGTGGACCTCG-3'.

Ad-Cre infections

Recombinant adenovirus encoding Cre recombinase (Ad-Cre) was purchased from the University of Pennsylvania Gene Transfer Vector Core. 6 to 8 week-old mice were infected intranasally with 100 µl of saline containing 3×10^{10} PFU Ad-Cre. 20 minutes prior to injection of 0.5 µl of 2 mol/l CaCl₂ was added to improve gene transfer to airway epithelia. Before injections mice were anesthetized with ketamine (100mg/kg).

Histology and immunohistochemistry

For histological evaluation, whole lungs were manually inflated and fixed with 10% formalin and then embedded in paraffin blocks. Longitudinal sections of lung were stained with hematoxylin and eosin (H&E) and evaluated by Dr. Steven Albelda (University of Pennsylvania). Tumors were given histologic scores as 0 (minimal tumors seen), 1 (small patchy areas of tumors), and 2 (extensive lung tumors).

***pTRE-PKCε* vector construction**

cDNA encoding for PKCε was excised from the pB.PKCε vector and ligated into a pTRE-tight vector using T4 DNA ligase (Life Technologies). Initially, the pB.PKCε vector was modified by inserting a linker containing *Mlu*I, ATG and HA epitope using restriction enzymes *Not*I and *Xho*I (New England Biolabs). This modification generated a sequence coding for Mlu-ATG-HA-XhoI- PKCε- Mlu in the pB.PKCε vector. For the pTRE-tight vector, a stop codon was added flanked with *Mlu* and *Not*I restriction sites. Finally, the ATG-HA-PKCε was released from the pB.PKCε vector by digesting with *Mlu* and ligated into the pTRE-tight vector. pTRE-PKCε plasmid was transformed into *E.coli* (DH5α) and was purified using QIAprep maxi kit (Qiagen) according to the manufacturer's instructions. The purified plasmid was digested with the restriction enzyme *Mlu* to confirm isolated clones for cDNA insert and the plasmid vector. Clones with the correct orientation of insertion were selected after sequencing and verified by vector mapping using various enzymes. For *in vitro* validation of PKCε expression, Cos-1 cells (1×10^5 cells/well in 12-well plates) were transfected with 0.1 µg/µL of pTet-On and 0.4 µg/µL of pTRE-PKCε using Lipofectamine2000 (Invitrogen). After 24 h, cells were treated with doxycycline (Clontech) at concentrations of 0.1, 1, 5 and 10 µg/µL. The expression of PKCε was also validated by transfecting pTRE-PKCε. pTRE-PKCε was digested and linearized by *Pci*I and *bcl*I to remove unnecessary backbone of the pTRE-tight vector and was submitted to the Wistar Institute Transgenic Mouse Facility for pronuclear injection. Chimeric mice were generated in S129/B6 background.

Southern blot

Genomic DNA was digested overnight with *BglII* and subjected to electrophoresis on a 0.7 % agarose gel. DNA was transferred to Zeta-Probe GT (Biorad) nylon membrane. The membrane was UV crosslinked and prehybridized in ExpressHyb solution (Clontech) containing salmon sperm DNA at 60⁰ C. The 239 base pair fragment of the plasmid pB.PKCε digested with *PstI* and *BamHI* was used as probe, radiolabeled using Exo (-) Klenow DNA polymerase (Biorad) and hybridized with DNA on the membrane in ExpressHyb solution overnight at 60⁰ C. The membrane was exposed to X-ray film and visualized by autoradiography.

CHAPTER 5: Conclusions and Future Directions

This thesis work established the importance of PKC isozymes in the initiation and progression of lung cancer. PKC isozymes were found to regulate drug resistance, NF- κ B pathway activation, and KRAS-driven lung tumorigenesis. We showed for the first time that PKC isozymes are implicated in the selective and adaptive mechanisms that are responsible for acquired resistance to molecular-targeted therapy. Our results indicate altered expression of PKC α and PKC δ in NSCLC cells resistant to erlotinib. Furthermore, overexpression of PKC α and down-regulation of PKC δ are required to induce erlotinib resistance. We also provided data showing that PKC α is essential for the maintenance of mesenchymal phenotype observed in erlotinib-resistant cells. Similar to drug resistance, inflammation is another key factor that contributes to the progression of lung cancer. Our data indicate that PKC α is a positive regulator of NF- κ B signals in NSCLC cells, suggesting its role in the promotion of inflammation. PKC α regulates key steps of the canonical NF- κ B pathway activation, including I κ B α phosphorylation, NF- κ B promoter activity, NF- κ B DNA binding, and expression of NF- κ B responsive genes involved in cell survival, motility, angiogenesis, and inflammation. The role of PKCs in lung cancer is not limited to disease progression, as we also identified the requirement for another PKC isozyme, PKC ϵ , in the development of KRAS-driven lung tumor formation. By generating a mouse model that expresses mutant KRAS in a null PKC ϵ background, we demonstrated that PKC ϵ is required for KRAS-driven lung tumorigenesis.

Resistance to therapy has been a major hurdle in lung cancer treatment. Identification of mechanisms associated with acquired resistance presents a strategy to overcome this hurdle. Our data in Chapter 2 provides compelling evidence for the use of

PKC α inhibitors in combination with erlotinib. Inhibition of PKC α using pharmacological inhibitors or with RNA interference significantly enhanced the sensitivity of NSCLC cells to erlotinib. PKC α was also shown to modulate its effect on erlotinib sensitivity, in part, by regulating the expression of PKC δ . Overexpression of PKC α in erlotinib-sensitive cells led to down-regulation of PKC δ , suggesting that the low levels of PKC δ seen in the erlotinib-resistant cells could have been driven by the overexpression in PKC α . Furthermore, reconstitution of PKC δ in erlotinib-resistant cells enhanced their sensitivity to erlotinib, thus underscoring the importance of PKC δ down-regulation in acquired resistance to erlotinib therapy.

Chapter 2 of this thesis also provides insight into how PKC α could be implicated in the selective mechanism that induces resistance to erlotinib. As previously documented, erlotinib-resistant cells have elevated expression of genes associated with EMT and display morphological changes that are reminiscent of the mesenchymal phenotype [212]. Our results demonstrated that although PKC α does not affect the expression of the epithelial marker E-cadherin, it regulates the expression of genes implicated in the induction of the mesenchymal phenotype, such as vimentin and Snail. The mechanisms through which PKC α acts to regulate the expression of genes associated with mesenchymal phenotype in lung cancer cells are yet to be determined. Recent studies in breast cancer revealed that PKC α preferentially supports the maintenance of mesenchymal cell state through its regulation of the transcription factor FRA1 (Fos-related antigen 1). Whether a similar mechanism operates in lung cancer remains to be determined. A potential transcription factor that could be involved in PKC α -dependent

expression of EMT markers in NSCLC cells is NF- κ B. Studies have identified known EMT marker genes such as vimentin, Snail and Zeb2 as target genes of NF- κ B in several cancer types [9, 33, 103, 200] and in Chapter 3 of this thesis, we have established that PKC α -mediated regulation of NF- κ B is required for the expression of NF- κ B responsive genes in NSCLC cells. It would be important to determine if PKC α -dependent NF- κ B activation is required for the transcription of vimentin, Snail, Twist and Zeb2 in erlotinib-resistant cells.

Our results also indicate that a subpopulation of erlotinib naive cells that express the mesenchymal surface marker CD44 exhibit higher levels of PKC α expression compared to the cell population where CD44 is absent. It would be interesting to see if stable overexpression of PKC α could increase the expression of CD44 as well as the expression of genes associated with the mesenchymal phenotype. Several studies have shown that cells undergoing EMT become aggressive and develop resistance to therapies. Conversely, EMT can be induced as a consequence of exposure to therapeutic drugs [182]. Our model of erlotinib-resistant cells presents yet a different mechanism for drug resistance where EMT and drug resistance co-exist, but do not have a causal relationship. As previously reported [212], depletion of genes associated with the mesenchymal phenotype did not alter the sensitivity to erlotinib, and erlotinib naive cells possess a subpopulation that already underwent EMT.

The cells we used as a model for erlotinib resistance have high levels of TGF- β compared to the parental erlotinib-sensitive cells. TGF- β signaling is required and sufficient for the induction of erlotinib resistance and EMT in erlotinib-resistant cells and

our results revealed that TGF- β signaling drives the expression of PKC α in these cells. To assert the explicit dependence of PKC α expression on TGF- β , it is important to determine if inhibiting TGF- β levels in erlotinib-resistant cells to the level observed in the parental erlotinib-sensitive cells could decrease PKC α expression. Although the mechanism by which overexpression of PKC α mediates erlotinib resistance and EMT remains to be determined, we propose a model implicating PKC α in the transition from epithelial to mesenchymal cells and drug resistance in NSCLC cells (Fig. 5.1).

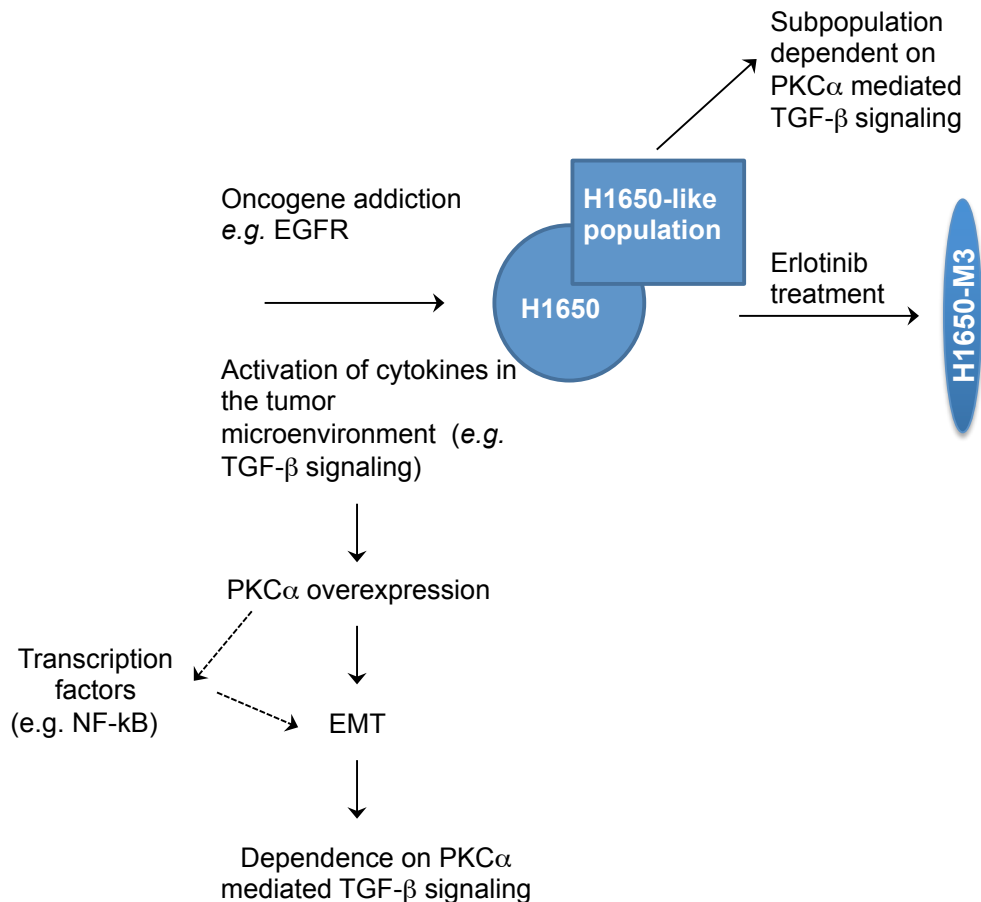


Figure 5.1 Proposed model for PKC α -mediated erlotinib resistance in NSCLC cells

In Chapter 3, we demonstrated an additional role of PKC α in cellular processes implicated in lung cancer progression. PKC α regulates the activation of the NF- κ B pathway in response to IL-1 β . Inhibition of PKC α resulted in decreased I κ B α phosphorylation, indicating that it regulates the canonical NF- κ B pathway. The effect was further supported by the observation that PKC α inhibition blocks NF- κ B translocation and DNA binding. Activation of NF- κ B is required for the induction of genes in response to IL-1 β including pro-inflammatory genes. Our findings indicate that PKC α -mediated activation of NF- κ B by IL-1 β results in up-regulation of NF- κ B-responsive genes such as MMP9, COX-2 and IL-8. These findings suggest that PKC α could play an important role in promoting lung cancer progression that results from chronic inflammation.

Future experiments must be done to identify the mechanism for PKC α -mediated activation of NF- κ B in response to IL-1 β . PKCs translocate from the cytosol to the plasma membrane upon activation. We can use real-time microscopy to assess whether IL-1 β could induce changes in the subcellular localization of PKC α using a GFP-fused PKC α construct. If PKC α translocates to the plasma membrane upon IL-1 β stimulation, it could potentially associate with IL-1R, the receptor for IL-1 β . Indeed, an analogous mechanism was recently discovered by our laboratory for PKC ϵ and the receptor for TNF α [54]. Immunoprecipitation of IL-1R from H460 cells will show whether endogenous PKC α coimmunoprecipitates with this receptor. If PKC α associates with the IL-1R, we speculate that IL-1 β treatment will enhance that association. PKC α could also

be involved in the activation of NF- κ B through phosphorylation of IKKs or phosphorylation of the RelA subunit important for the localization of the p50/RelA dimer to the nucleus. *In vitro* kinase assay could be utilized to determine if PKC α directly phosphorylates the IKK complex or the RelA subunit in NSCLC cells. These potential mechanisms are summarized in Fig. 5.2.

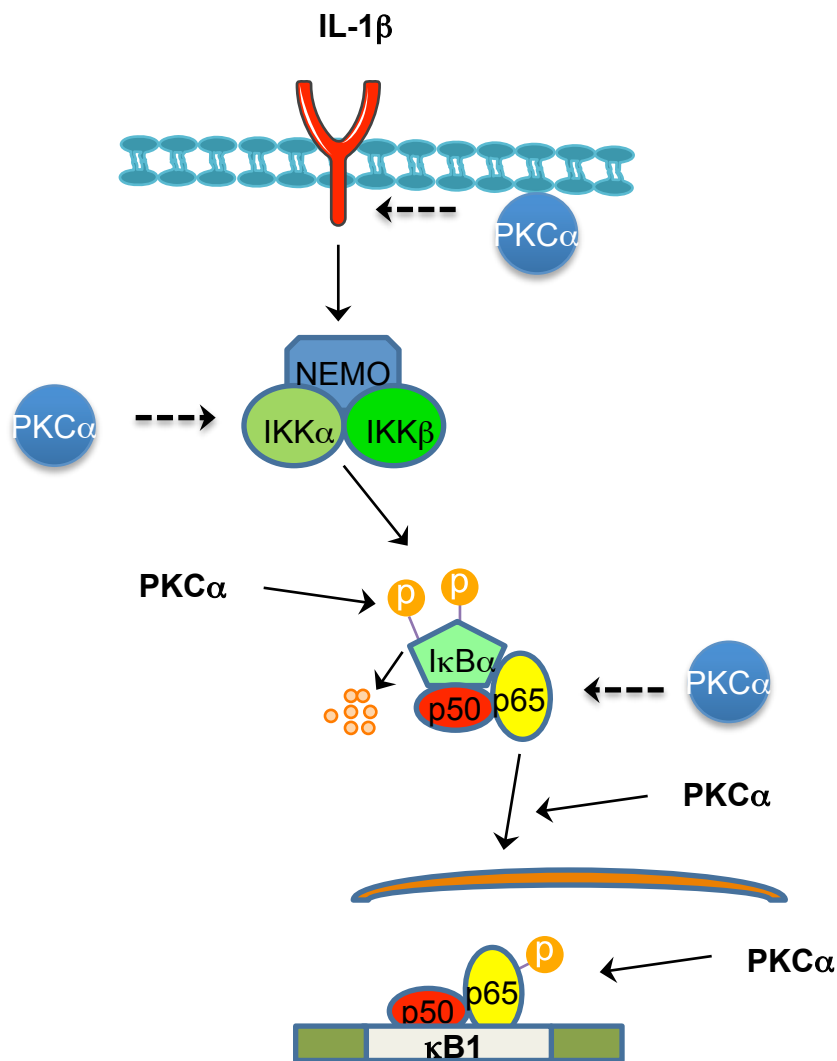


Figure 5.2 Proposed model for the regulation of the canonical NF- κ B pathway by PKC α in NSCLC cells.

The work presented in this thesis also highlights the importance of PKC isozymes in lung cancer initiation. Previous studies from our lab established a key role for PKC ϵ in lung cancer progression in KRAS mutant NSCLC cell lines and xenograft models [25, 26]. In Chapter 4, we further demonstrated the requirement of PKC ϵ for KRAS-induced lung tumor formation in a mouse model where mutant KRAS is expressed in a PKC ϵ -null background. Indeed, genetic ablation of PKC ϵ significantly attenuated tumor formation in KRAS mutant mice. Future experiments include determining what role PKC ϵ plays in the maintenance of KRAS-induced lung tumors, as well as what mechanisms and downstream effectors of PKC ϵ mediate KRAS signaling.

It would also be important to determine whether overexpression of PKC ϵ accelerates KRAS-driven lung tumor formation, and if by itself, is sufficient to induce precancerous or cancerous lesions in the lungs. We generated a mouse model that specifically expresses PKC ϵ in the lungs when crossed with SPC-rtTA transgenic mice. Future experiments could use this model to generate KRAS mutant mice that overexpress PKC ϵ in the lungs. We speculate that overexpression of PKC ϵ in the lungs leads to the formation of preneoplastic and neoplastic lesions. On the basis of our preliminary results from bitransgenic *KRAS*^{G12D};*PKC ϵ* ^{-/-} mice, we also expect that overexpression of PKC ϵ accelerates tumor formation and increases tumor incidence in the lungs.

In conclusion, the data presented in this thesis has further established the significance of PKC isozymes in lung cancer development. Our work has identified the requirement of PKC α in selective and adaptive processes that result in the loss of

erlotinib sensitivity. We further established the importance of PKC α in the progression of NSCLC by demonstrating its regulation of the NF- κ B pathway. Our results support the use of PKC α inhibitors for lung cancer patients with indications of TKI resistance and hyperactivation of the NF- κ B pathway. Additionally, our work gives insights into the role of PKC isozymes in the initiation of lung cancer. We identified the requirement of PKC ϵ signaling in KRAS-driven lung tumor formation. Pharmaceutical companies have been interested in the development of PKC ϵ specific inhibitors for cancer treatment [38] and our results corroborate that PKC ϵ specific inhibitors can be effective candidates for lung cancer prevention and treatment.

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